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(54) Title: MATERIALS AND METHODS RELATING TO THE MODULATION OF T CELL RESPONSE TO SOLUBLE ANTIGEN

(57) Abstract: The invention relates to materials and methods for improving vaccination strategies. Particularly, but not exclusively, the present invention relates to the use of an activator of NKT cells, in combination with an activator of Toll-like receptors, to enhance T-cell responses to soluble antigen. The invention also relates to the use of particular NKT cell activators, e.g. OCH, to enhance T-cell responses to soluble antigen. The materials and methods of the invention are particularly suitable for enhancing presentation of the soluble antigen via the MHC I molecules of dendritic cells and so stimulating cytotoxic T cell responses against the antigen.

Materials and Methods Relating to the Modulation of T cell response to soluble antigen

Field of the invention

The present invention relates to materials and methods for improving vaccination strategies. Particularly, but not exclusively, the present invention relates to the use of an activator of NKT cells as an adjuvant to enhance T-cell responses to soluble antigen.

Background of the invention

A significant challenge for the immune system is to be able to distinguish between harmful and benign antigenic stimuli in order to avoid inappropriate adaptive responses. Evidence suggests that modification in the function of antigen-presenting cells (APC), specifically dendritic cells (DC), plays a critical role in this process (1). DC reside in peripheral tissues in an immature state defined by significant capacity for antigen uptake and low expression of MHC and costimulatory molecules. Upon exposure to inflammatory stimuli, DC undergo a maturation process involving migration to lymphoid sites, down-regulation in rate of antigen uptake and increase in levels of MHC and costimulatory molecules. These changes enable DC to present antigens to naive $CD4^+$ and $CD8^+$ T cells and thereby promote adaptive immune responses (2).

The potency of DC as APC for naïve T cells can also be modulated directly by microbial stimuli via pattern recognition receptors, of which toll-like receptors are the prime examples (3), and also by signals such as CD40L (4, 5), RANKL (6) and FasL (7) mediated by T cells themselves. With respect to the latter T 'helper' function, studies have shown that $CD4^+$ T cells can activate DC specifically through signals

mediated via CD40, inducing further upregulation of surface molecules involved in T cell stimulation and inducing the release of IL-12 (5). This 'conditioning' of DC is particularly important for the generation of CTL from naïve $CD8^+$ T cell precursors (8-10). It has been reported that maturation of DC in the absence of CD40 signalling can diminish the ability of these cells to release IL-12 (11), and therefore potentially weakens their stimulatory capacity for CTL. T cell help is therefore most likely to be effective if it is achieved through interaction with immature DC early in the evolution of a T cell-mediated response.

Exogenous protein antigens can be taken up by APC and processed into peptides for presentation on the cell surface by MHC class II molecules to $CD4^+$ T cells. This has been referred to as the "exogenous" pathway of antigen presentation. In contrast, internal antigens are processed and presented by MHC class I molecules to $CD8^+$ T cells; the so-called "endogenous" pathway of antigen presentation (18, 19). It is now becoming clear that some APC, including subsets of DC, are capable presenting peptides derived from exogenous antigens on MHC class I molecules, resulting in the stimulation of $CD8^+$ T cell responses (20-22). This would be a desirable outcome for protein-based vaccine development in which CTL responses are sought.

However, such "cross presentation" is generally thought to be an inefficient process, and in many instances has led to tolerance rather than immunity (reviewed in (1, 23). It has been reported that the provision of T cell help can induce immunity from a previously tolerogenically cross-presented antigen (24, 25).

Another obstacle to the efficacy of help provided by T cells in this manner is the scarcity of naïve T cells with reactivity to unique antigens presented by the DC.

An alternative source of help is NKT cells, αβ T cells with an invariant TCR and intermediate level NK1 surface expression. CD1d-restricted NKT cells expressing the invariant TCR α chain encoded by Vα14Jα281 gene segments in mice, and Vα24JαQ in humans, (NKT cells) are found in relative abundance in tissues such as spleen, bone marrow, thymus and liver (12, 13).

Although the ligand for such invariant TCR cells has not been defined, it is possible that NKT cells respond to a common glycolipid presented by CD1d molecules that is induced as an innate response to cellular distress. As NKT can express CD40L and secrete significant levels of inflammatory cytokines, it is possible that these cells can be readily induced to activate DC (14). Recent studies have shown that human NKT cell clones restricted to the group I CD1 molecules CD1a, CD1b and CD1c, and γδ T cells restricted by CD1c, can directly induce maturation of cultured DC in vitro (16, 17).

Injection of purified DC has been used to obtain T-cell response to antigen, but only in immunisation strategies using short peptide antigens, not whole protein antigen (15). Using whole antigen is preferable as it enables a range of peptides to be presented, invokes both CD4+ and CD8+ T-cell responses and may stimulate the secretion of protein specific antibodies. In addition, the use of purified cells for vaccination is a lengthy and complex procedure and not practical for use in actual vaccination protocols. There is thus a continuing need in the art for methods of improving T-cell response to foreign antigen, particularly methods which improve 'cross presentation' of antigen and thus lead to CD8+ T cell response as well as CD4+ cell response and antibody secretion.

Summary of the invention

The inventors have investigated whether NKT cells can influence immunological responses to soluble antigen in the presence of α-galactosyl ceramide (α-GalCer), a CD1d-binding glycolipid that has been shown to specifically induce activation of NKT cells (26). The inventors show that, while intravenous and oral administration of ovalbumin protein alone can induce activation of $CD4^+$ and $CD8^+$ T cells, these responses are considerably enhanced by concomitant activation of NKT cells leading to modulation of DC function.

The inventors have further shown that the enhanced immune response observed after stimulating NKT cells can be significantly further enhanced by stimulating simultaneously Toll Like Receptors (TLR) expressed on DC and B cells. TLR are pattern recognition receptors which bind bacterial products to alert the immune system of the presence of bacteria in the body.

Stimulation of NKT cells leads to the maturation of DC upon the interaction of CD40L (expressed by activated NKT cells) and CD40 (expressed by DC). The inventors have found that stimulation of TLR4 has a synergistic effect with the CD40 dependent stimulation pathway and results in a much stronger immune response. TLR4 can be stimulated by LPS and a derivative of LPS "MPL", which is non-toxic and can be injected into patients.

Injection of α-GalCer has been used to enhance anti-tumour immune responses, chiefly by activating NKT cells which kill tumour cells in an NK-like way (47) or by activating NK cells themselves (51). It has also been reported that the anti-tumour effect of α-GalCer may in part be due to stimulation of CD8+ T cells by IFNγ produced by activated NKT cells (50). WO03/009812 suggests the use of glycosylceramides, including

α-GalCer, as activators of NKT cells in immunisation protocols designed to elicit a T-cell response.

As described above, isolated and purified DC cells loaded with antigenic peptide may be used for immunisation. In some studies, peptide-expressing DC cells have been treated with α-GalCer before reinjection (15).

However, this immunisation approach is of limited applicability in practice. The multiple steps involved in generation of DC (usually from bone marrow cells), culturing the cells, loading with peptide and re-injecting into the host are time-consuming and complex. In addition, using peptide antigen means that only CD8+ or CD4+ T-cell responses are invoked, depending on the MHC specificity of the peptide. It also requires that the antigenic epitope of whatever antigen it is desired to raise an immune response against be known.

The method of the present invention has several advantages. Firstly, whole antigen is used for vaccination, so both CD4+, CD8+ T-cell and antibody responses are obtained, resulting in an enhanced T-cell response overall. Secondly, the strategy is much more simple then existing strategies as it does not require that DC cells be isolated, cultured or reinjected. Thirdly, it requires preparation of only the soluble antigen - the antigenic peptide need not be identified or purified. Fourthly, it can be used with any individual, independent of MHC haplotype.

Accordingly, at its most general, the invention provides materials and methods for inducing an immune response to soluble polypeptide antigen using an NKT cell activator.

In a first aspect of the present invention, there is provided a composition for inducing an immune response in an individual

to an antigen, said composition comprising said antigen in combination with an activator of NKT cells.

The invention also provides the use of an activator of NKT cells in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual.

The invention also provides for a kit having first and second containers, wherein the first container comprises a composition comprising an activator of NKT cells, and the second container comprises a composition comprising a soluble polypeptide antigen.

The activator of NKT cells may be a glycolipid presented by CD1d molecules or any molecule which activates NKT cells. Activation of NKT cells may be detected by, for example, increased expression of CD40L or secretion of cytokine such as IFNγ or IL-4.

The activator of NKT cells may be a natural ligand of NKT cells. Alternatively, the activator of NKT cells may be α-GalCer, or an analogue of α-GalCer such as OCH (described in ref. 30) or alpha-glucosyl ceramide, or it may be a TCR specific antibody. Methods for synthesis of α-GalCer are described by Figueroa-Perez and Schmidt, Carbohydrate Research 328 (2000) 95-102, and in US 6,555,372.

The immune response is preferably a T cell response. It may be a CD4+ T cell response or CD8+ T cell response. Preferably, the immune response is both a CD4+ and a CD8+ T cell response. The immune response may also be an antibody response.

The antigen may be any antigen of choice, but is preferably a soluble protein.

The composition may further comprise a TLR activator, that is to say a substance which activates a Toll-like receptor. It is believed that use of TLR activators in conjunction with activators of NKT cells provide a synergistic adjuvant effect. Preferably, the TLR activator is an activator of TLR3, TLR4, TLR5, TLR7 or TLR9. A suitable TLR activator is MPL (monophosphoryl lipid A), which binds TLR4. Other TLR activators which may be used are LTA (lipoteichoic acid) and PGN (peptidoglycan), which bind TLR2 and TLR6; Poly I:C (polyinosine-polycytidylic acid), which binds TLR3; flagellin, which binds TLR5; imiquimod (1-(2-methylpropyl)-1*H*-imidazo(4,5-c)quinolin-4-amine), which binds TLR7 and CpG (DNA CpG motifs), which binds TLR9; or any other component which binds and activates a TLR. For more details, see Caetano Reis e Sousa, Toll-like receptors and dendritic cells. Seminars in Immunology 16:27, 2004.

However, use of TLRs in accordance with the invention is not restricted to use in compositions comprising NKT cell activators. The skilled person will appreciate that the NKT cell activator and the TLR activator may be administered in separate compositions.

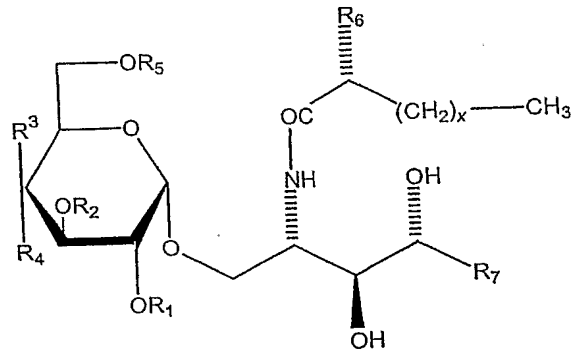
Thus the invention provides the use of an activator of NKT cells in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with a TLR activator.

Also provided is the use of a TLR activator in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with an activator of NKT cells.

In these aspects of the invention, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

The term glycosylceramide is used herein to refer to compounds of the formula I as disclosed in WO03/009812.

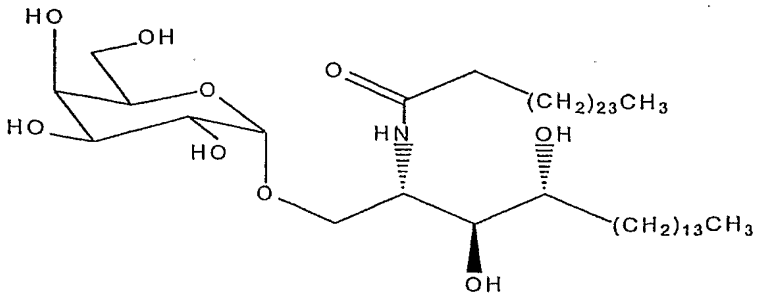
Formula I:



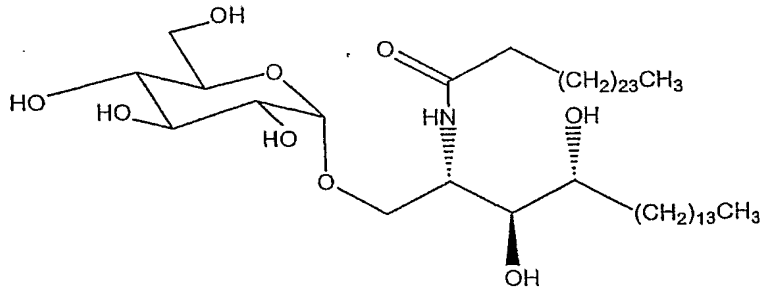
wherein R_1, R_2 and R_5 represent H or a specific monosaccharide; R_3 and R_6 represent H or OH, respectively; R_4 represents H, OH or a specific monosaccharide; X denotes an integer from 1 to 23; R_7 represents any one of the following groups (a)-(g): (a) $-(CH_2)_{11}-CH_3$, (b) $-(CH_2)_{12}-CH_3$, (c) $-(CH_2)_{13}-CH_3$, (d) $-(CH_2)_9-CH(CH_3)_2$, (e) $-(CH_2)_{10}-CH(CH_3)_2$, (f) $-(CH_2)_{11}-CH(CH_3)_2$, (g) $-(CH_2)_{11}-CH(CH_3)-C_2H_5$.

The activator of NKT cells may be α-GalCer, OCH, α-glucosylceramide, or any of the other NKT activators referred to in this specification. αGalCer, α-glucosylceramide and OCH have the following formulae:

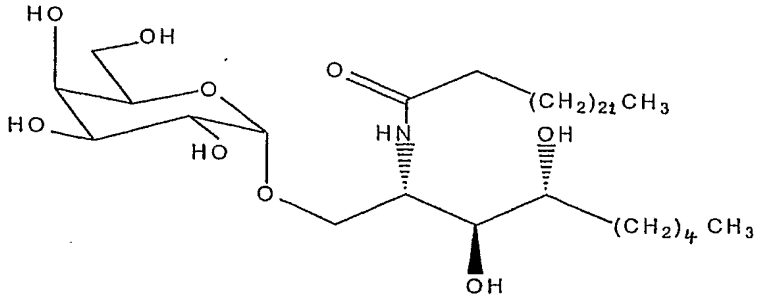
α-GalCer



α-glucosylceramide



OCH



The TLR activator may be an activator of TLR3, TLR4, TLR5, TLR7 or TLR9, including any of the specific activators referred to above.

The invention further provides the use of α-GalCer in the preparation of a medicament for inducing an immune response to

The invention further provides the use of α-GalCer in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with CpG or MPL.

The invention further provides the use of α-glucosylceramide in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with CpG or MPL.

In a further aspect the invention provides a composition comprising an activator of NKT cells and a TLR activator, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

Also provided is a composition comprising α-GalCer and MPL or CpG, and, further, a composition comprising α-glucosylceramide and MPL or CpG.

The compositions of the invention may further comprise a purified soluble polypeptide antigen.

Also provided is a composition comprising OCH and a purified soluble polypeptide antigen.

In further aspects, the invention provides kits containing combinations of some or all of NKT cell activators, TLR activators and soluble antigens.

Thus the invention provides a kit having first and second containers, wherein the first container comprises a composition comprising an activator of NKT cells, and the second container comprises a composition comprising a TLR

activator, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

Also provided is a kit having first and second containers, wherein the first container comprises a composition comprising α-GalCer, and the second container comprises a composition comprising CpG or MPL.

Also provided is a kit having first and second containers, wherein the first container comprises a composition comprising α-glucosylceramide, and the second container comprises a composition comprising CpG or MPL.

The kit may additionally have a third container, which container comprises a composition comprising a purified polypeptide antigen.

Also provided is a kit having first and second containers, wherein the first container comprises a composition comprising OCH, and the second container comprises a composition comprising a purified polypeptide antigen.

In the kits of the invention, any or all of said compositions may further comprise a pharmaceutically acceptable carrier or diluent.
Thus, for example, the invention provides for a kit having first, second and third containers, wherein the first container comprises a composition comprising an activator of NKT cells, the second container comprises a composition comprising a soluble polypeptide antigen, and the third container comprises a composition comprising a TLR activator.

The compositions of the invention, whether or not provided as part of kits, are preferably pharmaceutical compositions and,

if necessary, further comprise a pharmaceutically acceptable carrier or diluent. In one embodiment, the pharmaceutical composition may comprise a liposome which helps to target the peptide antigen to particular cells or tissue, e.g. lymphoid tissue, and increases the half life of the antigen. Liposomes include emulsions, foams, micelles etc.

The pharmaceutical composition may also comprise at least one component which assists in the induction of any immune response. The one or more components may include lipids, e.g. palmitic acid residues attached to the peptide antigen, or E.coli lipoproteins such as tripalmitoyl-S-glycerylcystein lyseryl-serine (P_3 CSS); and adjuvants, e.g. Freund's adjuvant.

The pharmaceutical composition according to the invention may be used as a vaccine. The vaccine may comprise one or more protein, polypeptide or peptide antigens in combination with the NKT cell activator. The one or more peptides may be linked to a carrier such as thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus care protein etc.

Alternatively, the peptide may be present in the vaccine as a homopolymer comprising multiple copies of the same peptide, or as a heteropolymer of various peptides comprising antigens or epitopes of interest.

The vaccines may further comprise physiologically tolerable/acceptable diluent such as water or saline, e.g. phosphate buffered saline. Further, as mentioned above the vaccine preferably includes an adjuvant such as incomplete Freund's adjuvant, aluminium phosphate, aluminium hydroxide, or alum.

In a second aspect of the invention, there is provided a method of immunising an individual against an antigen, said method comprising the step of administering to said individual said antigen and a NKT cell activator, optionally in combination with a TLR activator as already described.

The activator of NKT cells and the antigen may be administered simultaneously, preferably as a composition according the invention. Alternatively, the activator of NKT cells may be administered before the polypeptide antigen is administered, preferably less than about 2 hours before. Likewise, the activator of NKT cells may be administered after the polypeptide antigen is administered, preferably less than about 8 hours after.

The vaccine composition may be administered via injection, aerosol, oral, transdermal, transmucosal, intrapleural, intrathecal, or other suitable routes, e.g. intravenously, subcutaneously, intradermally, or intramuscularly.

The present inventors have further determined that the enhanced immune response observed after stimulating NKT cells can be significantly further enhanced by stimulating simultaneously the TLR4, which is expressed on DC, and other TLRs. Therefore, in all aspects of the invention, an activator of a TLR receptor may be used in combination with the activator of NKT cells. A suitable TLR activator is MPL, which binds TLR4. Other TLR activators which may be used are LTA and PGN, which bind TLR2 and TLR6; Poly I:C, which binds TLR3; flagellin, which binds TLR5; imiquimod, which binds TLR7 and CpG, which binds TLR9; or any other component which binds and activates a TLR.

In one embodiment, the soluble antigen is a tumour antigen. Alternatively, the antigen may be a polypeptide unique to a

virus or bacterial cell, a parasite cell, or cellular products.

Peptides that comprises the antigen or epitopes of interest can be prepared synthetically, or by recombinant DNA technology or from natural sources such as tumours or viruses. Although the peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Description of the Figures

Figure 1. Intravenous injection of soluble antigen induces functional T cell responses that are enhanced by co-administration of α-GalCer.
Chicken ovalbumin suspended in PBS was injected into C57BL/6 mice (400 μg/mouse) together with either α-GalCer (1 μg/mouse) or vehicle. **(A)** Induction of $CD8^+$ T cell responses specific for the $OVA_{257-264}$ epitope was assessed in the blood by FACS analysis using fluorescent tetrameric H-$2K^b$/$OVA_{257-264}$ complexes. FACS profiles of naïve animals (control) and those receiving ovalbumin ± α-GalCer or vehicle are shown. Mean proportions of $tetramer^+$ cells as a percentage of $CD8^+$ cells (± SEM) for each treatment group are charted. **(B)** Thirteen days after ovalbumin administration, cytolytic activity of the induced $OVA_{257-264}$-specific response was assessed in vivo against various CFSE-labeled syngeneic splenocytes loaded with titrated doses of $OVA_{257-264}$-peptide as indicated, and a control splenocyte population without peptide labeled with CMTMR. Representative

FACS profiles for each treatment group are shown. Analysis of antigen specific lysis was calculated at 16 h after target cell administration, with percent specific lysis calculated as the mean proportion of antigen loaded cells depleted relative to control populations without antigen. **(C)** ELISpot assays were used to assess induction of IFNγ by $CD8^+$ cells responding to the MHC class I-binding peptide $OVA_{257-264}$, or $CD4^+$ T cells responding to the class II-binding peptides $OVA_{265-290}$ and $OVA_{323-339}$, 11 days after ovalbumin administration. For these analyses, splenocytes isolated from animals of the different treatment groups were stimulated with each of the peptides in vitro for 48 h.

Figure 2. Intravenous injection of soluble antigen ± α-GalCer induces functional T cell responses with potent restimulatory capacity.

$OVA_{257-264}$-specific T cell responses in groups of animals (n = 5) receiving intravenous injection with ovalbumin together with either α-GalCer or vehicle, were restimulated with either **(A)** i.v. injection of recombinant vaccinia encoding chicken ovalbumin, or **(B)** i.v. injection of DC loaded with $OVA_{257-264}$-peptide. Specific responses were assessed in the blood by FACS analysis using fluorescent tetrameric $H\text{-}2K^b/OVA_{257-264}$ complexes seven days after restimulation. Charts showing mean responses for each treatment group (± SEM) prior to restimulation (left panels) and after restimulation (right panels) are presented. Control groups without prior ovalbumin treatment served to control for efficacy of the secondary stimulus, and naïve animals, or animals treated with DC without peptide, served as negative controls.

Figure 3. Enhancement of T cell responses to soluble antigen with injection of α-GalCer requires the involvement of NKT cells.

$OVA_{257-264}$-specific T cell responses were assessed in the blood using $H\text{-}2K^b/OVA_{257-264}$ tetramers in groups (n = 5) of wildtype

C57BL/6 animals and **(A)** $NKT^{-/-}$ mice, or **(B)** $CD1d^{-/-}$ mice, seven days after receiving intravenous injection with ovalbumin together with either vehicle or α-GalCer. **(C)** $OVA_{257-264}$-specific T cell responses were likewise assessed in wildtype animals receiving vehicle, α-GalCer or β-GalCer, a glycolipid known to bind CD1d but unable to stimulate NKT cells (36).

Figure 4. Injection of α-GalCer enhances T cell responses to soluble protein antigen only if administered in close temporal association with protein.

$OVA_{257-264}$-specific T cell responses were assessed in the blood seven days after groups of animals (n = 5) received intravenous injection with ovalbumin (time zero = 0 h) and α-GalCer or vehicle injected at the indicated times relative to protein injection.

Figure 5. DC-induced T cell responses can be enhanced by concomitant, α-GalCer-mediated, activation of NKT cells capable of substituting for $CD4^+$ T cell help.

(A) LCMV GP_{34-41}-specific $CD8^+$ T cell responses were assessed in the blood of groups of animals (n =5) seven days after they had received DC loaded with LCMV GP_{34-41} together with α-GalCer, vehicle or PBS, or untreated DC. One group (first panel) received DC that were loaded with LCMV GP_{34-41} and α-GalCer separately, and then combined before injection. In some instances, $NKT^{-/-}$ mice were used as recipients (right panel), and in others the DC were matured with LPS prior to injection (lower panel). **(B)** $OVA_{257-264}$-specific T cell responses were assessed in animals that received α-GalCer- or vehicle-treated DC loaded with the MHC class I-binding peptide $OVA_{257-264}$ alone, or together with the class II-binding peptide $OVA_{323-339}$. Animals injected with DC without peptides served as negative controls. **(C)** $OVA_{257-264}$-specific T cell responses were assessed in animals that received α-GalCer- or vehicle-treated DC exposed to whole ovalbumin for 20 h. Animals injected with DC without protein served as negative controls.

Figure 6. Injection of α-GalCer leads to maturation of splenic dendritic cells and increases cross presentation. Antibody conjugated to magnetic beads was used to isolate $CD11c^+$ cells from the spleens of animals that had received i.v injection of ovalbumin together with vehicle, α-GalCer or LPS 24 h previously. Representative FACS profiles showing expression of CD80 and CD86 on positively sorted $CD11c^+$ cells from ovalbumin ± α-GalCer treated animals relative to levels on $CD11c^+$ cells from naïve controls (grey).

Figure 7. Activation of NKT cells enhances T cell responses to soluble protein via a CD40-mediated mechanism independent of IFNγ production.
$OVA_{257-264}$-specific T cell responses were assessed in the blood seven days after groups (n = 5) of C57BL/6 or $CD40L^{-/-}$ mice received intravenous injection with ovalbumin together with either vehicle or α-GalCer **(A)**, with serum cytokine levels in α-GalCer-treated animals determined by ELISA at the indicated times **(B)**. $OVA_{257-264}$-specific T cell responses were similarly assessed in $IFN\gamma R^{-/-}$ animals and 129S6/SvEv controls **(C)**, or in C57BL/6 animals injected with ovalbumin and the α-GalCer analogue OCH **(D)**. Serum cytokine levels were determined for the latter experiment **(E)**.

Figure 8. Oral administration of soluble antigen and alpha-GalCer induces functional CTL. (a) Whole OVA was administered by gavage (30 mg/mouse) together with either alpha-GalCer (8 μg/mouse) or vehicle. Induction of $OVA_{257-264}$-$CD8^+$ T cell responses was assessed in the blood 7 days later. (b) Cytolytic activity of the induced $OVA_{257-264}$-specific response was assessed in vivo 11 days after OVA administration against various CFSE-labeled syngeneic splenocytes loaded with titrated doses of $OVA_{257-264}$-peptide as indicated, and a control splenocyte population without peptide labelled with CMTMR.

Representative FACS profiles for each treatment group are shown. Analysis of antigen specific lysis was calculated at 16 h after target cell administration, with percent specific lysis calculated as the mean proportion of antigen loaded cells depleted relative to control populations without antigen.

Figure 9. Co-stimulation of TLR4 on DC further enhances T-cell response to soluble protein

Liposomes containing alphaGalcer + ovalblumin + MPL (an LPS derivative which binds TLR4) were injected i.v. into mice and 7 days later frequency of OVA specific CTL was measured by ex-vivo tetramer staining. Liposomes containing either ovalbumin and MPL or ovalbumin and alphaGalceramide were used as control. FACS profiles of naïve animals and those receiving ovalbumin +/- α-GalCer and/or MPL are shown. Mice immunized with liposomes containing OVA alone showed: 0.1% of tetramer+ cells; OVA + alphaGalcer: 2% of tetramer+ cells; OVA+alphaGalcer+MPL: 13% of tetramer+ cells.

Figure 10. Synergistic effect of several TLR ligands in combination with alphaGalactosylceramide in inducing mature DC.

Injection of iNKT cell ligands leads to maturation of splenic DCs, increasing their immunostimulatory capacity. Animals were treated i.v. with either 100 ng of iNKT cell ligand alpha-GalCer, or 25 μg of TLR4 ligand monophosphoryl lipid A (MPL), or 2.5 μg.poly I:C, or 2.5 μg.flagellin, or 50 μg.CpG. Surface expression of CD86 was assessed on splenic $CD11c^+$ cells 24 h later.

Figure 11. Activation of NKT cells enhances T cell responses to tumour-specific antigen.

Progression of EG.7OVA tumours implanted subcutaneously were monitored in animals that were subjected to; (i) no treatment, (ii) 400 μg ovalbumin together with vehicle 8 days prior to challenge (iii) 400 μg ovalbumin together with 1 μg α-GalCer 8 days prior to challenge. Mean tumour sizes per group (n = 5) ± SEM are shown.

Figure 12. Injection of αOCH with MPL leads to further maturation of splenic dendritic cells and increases expansion of cytolytic OVA specific CTL. Antibody conjugated to magnetic beads was used to isolate $CD11c^+$ cells from the spleens of animals that had received i.v injection of ovalbumin together with vehicle, OCH or OCH with MPL 24 h previously. Representative FACS profiles showing expression of CD86 on positively sorted $CD11c^+$ cells are shown.

Figure 13. Co-stimulation of TLR4 on DC further enhances T-cell response to soluble protein and OCH
OCH + ovalblumin + MPL were injected i.v. into mice and 7 days later frequency of OVA specific CTL was measured by ex-vivo tetramer staining. A) FACS profiles of naïve animals and those receiving ovalbumin +/- OCH and/or MPL are shown. B) Mean proportions of $tetramer^+$ cells as a percentage of $CD8^+$ cells (± SEM) for each treatment group are charted.

Figure 14. Injection of OCH and MPL leads to maturation of splenic DCs, increasing their immunostimulatory capacity. Animals were treated i.v. with either 1 μg of OCH, or 25 μg of MPL, or OCH plus MPL. The immunostimulatory capacity of $CD11c^+$ cells isolated from the spleens of these group of animals was assessed by loading with influenza $NP_{366-374}$ peptide ex vivo, and then by adding splenocytes transgenic for the $NP_{366-374}$ specific T cell receptor. A) proliferation of CFSE labelled transgenic splenocytes is shown B) secretion of gamma interferon by transgenic splenocytes is shown.

Detailed Description of the Invention

Pharmaceutical Compositions

The NKT activators of the invention may be formulated in pharmaceutical compositions, and administered to patients in a variety of forms.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant or an inert diluent. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Such compositions and preparations generally contain at least 0.1wt% of the compound.

Parenteral administration includes administration by the following routes: intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraocular, transepithelial, intraperitoneal and topical (including dermal, ocular, rectal, nasal, inhalation and aerosol), and rectal systemic routes. For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, solutions of the compounds or a derivative thereof, e.g. in physiological saline, a dispersion prepared with glycerol, liquid polyethylene glycol or oils.

In addition to one or more of the compounds, optionally in combination with other active ingredient, the compositions can comprise one or more of a pharmaceutically acceptable

excipient, carrier, buffer, stabiliser, isotonicizing agent, preservative or anti-oxidant or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. orally or parenterally.

Liquid pharmaceutical compositions are typically formulated to have a pH between about 3.0 and 9.0, more preferably between about 4.5 and 8.5 and still more preferably between about 5.0 and 8.0. The pH of a composition can be maintained by the use of a buffer such as acetate, citrate, phosphate, succinate, Tris or histidine, typically employed in the range from about 1 mM to 50 mM. The pH of compositions can otherwise be adjusted by using physiologically acceptable acids or bases.

Preservatives are generally included in pharmaceutical compositions to retard microbial growth, extending the shelf life of the compositions and allowing multiple use packaging. Examples of preservatives include phenol, meta-cresol, benzyl alcohol, para-hydroxybenzoic acid and its esters, methyl paraben, propyl paraben, benzalconium chloride and benzethonium chloride. Preservatives are typically employed in the range of about 0.1 to 1.0 % (w/v).

Experimental Procedures

Mice

C57BL/6, 129S6/SvEv and $CD40L^{-/-}$ mice were from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, Maine. Also used were mice lacking the Jα281 TCR gene segment (27) which were devoid of $V\alpha14^{+}$ NKT cells while having other lymphoid cell lineages intact (referred to in the text as $NKT^{-/-}$ mice), $CD1d^{-/-}$ mice (28) and IFN□$R^{-/-}$ mice (29). All animals were maintained at the Biomedical Services Unit of John

Radcliffe Hospital by brother x sister mating; in vivo experimental protocols were performed according to institutional guidelines.

In vitro culture media and reagents

Unless otherwise stated all cultures were maintained in complete medium (CM) comprising of RPMI (Sigma-Aldrich, Dorset, England) supplemented with 2 mM glutamine, 1 % penicillin-streptomycin, 5 x 10^{-5} M 2-mercapto-ethanol (all Invitrogen Ltd, Paisley, UK) and 10 % fetal bovine serum (Globepharm, Guildford, England). Chicken ovalbumin grade VII was from Sigma-Aldrich. Peptides derived from chicken ovalbumin, including $OVA_{257-264}$ (binding to H-$2K^b$), $OVA_{323-339}$ and $OVA_{265-280}$ (both binding and I-A^b), and peptide $LCMV_{34-41}$ (H-$2K^b$) derived from lymphocytic choriomeningitis virus glycoprotein, were prepared in-house. The glycolipids α-galactosylceramide (α-GalCer) and β-galactosylceramide (β-GalCer) were from Kirin Brewery Co. Ltd, Japan and Fluka Chemie AG, Switzerland respectively. The glycolipids were solubilized in 0.5 % Tween/PBS, hereafter referred to as 'vehicle'.

Administration of soluble antigen

Ovalbumin protein was diluted in PBS and admixed with PBS-diluted glycolipid solutions (α-GalCer, β-GalCer or OCH), or PBS-diluted vehicle, immediately prior to injection into the lateral vein. Each animal received 400 μg protein and either 1 μg of glycolipid, or equivalent final volume of vehicle.

Immunization with antigen-loaded DC

Bone marrow cells from C57BL/6 mice were cultured in 20 ng/ml IL-4 and 20 ng/ml GM-CSF for 7 d. Cultures typically contained 70-100 % DC as determined by fluorescent staining with the anti-CD11c (BD Pharmingen, San Diego, CA). DC were loaded with peptide by incubation in CM containing 10 μM synthetic peptide for 2 h, or loaded with whole protein by incubation in CM containing 100 μg/ml protein for 24 h. DC were loaded with α-

GalCer by incubation in CM containing 100 ng/ml for 40 h. Cells were washed to remove excess Ag prior to i.v. injection (6 x 10^5 DC per animal).

Infection with recombinant vaccinia virus

Mice were infected with 10^6 plaque forming units of the recombinant virus VV-OVA (31), expressing full length chicken ovalbumin, by intravenous injection in PBS.

Monitoring CTL responses with MHC tetramers

Tetrameric H-2 $K^b/OVA_{257-264}$ peptide, or tetrameric H-2 $K^b/LCMV_{34-41}$ peptide complexes were prepared as outlined in Altman et al. (32), and used to stain fresh PBL isolated from the lateral tail vein. Approximately 5 x 10^5 PBL were suspended in 20 μl CM and incubated with 0.5 μg of tetramer complexes at 37 °C for 20 min. The cells were then incubated with anti-CD8α (BD Pharmingen) for 10 min at 4 °C, washed twice with PBS, and resuspended in PBS for FACS analysis. Cells were analysed with FACScan hardware and CellQuest software (BD Biosciences , Mountain View, CA).

ELISpot

Single cell suspensions were prepared from murine spleens by gently teasing tissue through nylon gauze with a syringe plunger. The cell suspensions were treated with RBC lysis solution (Puregene, Gentra Systems, Minneapolis, USA) to deplete red blood cells, and resuspended in CM. Analysis of murine IFNγ-production by graded doses of splenocytes in response to stimulation with 10 μM peptide for 48 h was performed on MultiScreen-IP high protein binding 96-well plates (Millipore Corporation, Bedford, MA, USA) using MabTech mouse IFNγ ELISpot kit according to the manufacturers instructions. For all ELISpot analyses, splenocytes from each sample were stimulated with 20 μg/ml Concanavalin A to serve as positive controls for ability to produce IFNγ.

In vivo cytotoxicity assay

Cytotoxicity was assessed on fluorescence-labeled syngeneic spleen cell populations loaded with specific antigen administered by i.v. injection. Groups of immunized mice (n = 5), and a non-immunized control group (n = 5) were injected with a mixture of four differentially labeled populations; a control population without antigen fluorescence-labeled with the dye CMTMR (10 μM), and three target populations labeled with CFSE at different concentrations (1.65 nM, 0.3 nM and 0.07 nM), and loaded with titrated doses of specific peptide (5 μM, 500 nM, 50 nM). Cytotoxicity was assessed by FACS analysis on blood taken from the lateral tail vein. The mean percent survival of peptide-loaded targets cells was calculated relative to antigen-negative controls. Cytotoxic activity was then expressed as percent specific lysis, calculated by the equation 100 - mean percent survival of peptide-loaded targets.

Phenotypic analysis of splenic DC

DC were isolated from the spleens of animals that had been injected with α-GalCer or vehicle by using anti-CD11c magnetic beads (Miltenyi Biotech, Gladbach,Germany) according to the manufacturer's instructions. The isolated cells were assessed for expression of CD8□, CD8˜ and CD86 with specific monoclonal antibodies (BD Pharmingen).

ELISA. Serum cytokine levels were determined by ELISA using commercial ELISA kits (R&D Systems, Minneapolis, USA) following the manufacturers instructions.

Tumour immunity assay

Eight days after administration of ovalbumin ± α-GalCer or vehicle, groups of mice (n = 5) were challenged with EG7.OVA cells expressing a cDNA encoding the chicken ovalbumin sequence (33). Challenge was with 1 x 10^6 tumour cells injected s.c. into the flank. Mice were monitored for tumour growth every 3-4 days, and tumour size for each group was calculated

as the mean of the products of bisecting diameters (± SEM). Measurements were terminated for each group when the first animal developed a tumour in excess of 200 mm^2.

Results

Administration of α-GalCer together with soluble antigen provides potent immune responses.

The possibility that NKT cells have the potential to influence the quality of adaptive immune responses was investigated in the context of T cell priming via intravenous delivery of soluble antigen. Immune responses were therefore monitored in C57BL/6 mice injected with chicken ovalbumin protein in the presence or absence of the NKT cell ligand α-GalCer. This glycolipid has been shown to rapidly induce the release of a NKT-cell derived cytokines, leading to a 'cytokine storm' characterized by high levels of IFNγ and IL-4 in the serum (26, 34). In agreement with these studies, co-administration of 1 μg of α-GalCer with 400 μg ovalbumin induced a substantial increase of IFNγ in the serum over a 48 h period. Of particular interest was the possibility that NKT cell mediated signals, be they soluble factors or cognate interactions, could lead to cross presentation of peptides derived from the administered ovalbumin protein to peptide-specific $CD8^+$ T cells, thereby perhaps providing a potent, specific, CTL response. For this reason, immune responses were initially screened in the blood using MHC class I/peptide tetramers capable of highlighting $CD8^+$ T cells reactive to the H-2 K^b-binding peptide $OVA_{257-264}$. The result of these analyses, presented in Figure 1A, indicated that co-administration of ovalbumin and α-GalCer led to a significant increase in $OVA_{257-264}$-specific $CD8^+$ T cells in the blood over animals given ovalbumin and vehicle (a polysorbate solution in which the glycolipid was solubilized). Repeated experiments indicated that co-administration of α-GalCer with ovalbumin led to between 5- and 10-fold increases in $OVA_{257-264}$-specific $CD8^+$ T

cells in the blood. Thus, while cross presentation of peptides derived from ovalbumin was observed in the absence of activation of NKT cells, the levels of cross presentation were markedly increased by concomitant stimulation of NKT cell activity.

The functional state of the induced ovalbumin-reactive $CD8^+$ T cells was investigated by assessing cytotoxic capacity, cytokine induction and ability to respond to further antigenic stimulation. Cytotoxic capacity was assessed against $OVA_{257-264}$ peptide-loaded syngeneic splenocyte targets that had been labeled with fluorescent dye and injected into the lateral vein 13 days after initial priming with ovalbumin ± α-GalCer. Specific lysis of the antigen-loaded population was monitored relative to a differentially labeled control splenocyte population that was not loaded with peptide. This assay, presented in Figure 1B, indicated that the ovalbumin-specific $CD8^+$ T cells induced were indeed capable of mediating specific cell lysis. In fact, cytotoxic activity was observed in all ovalbumin treated animals regardless of whether α-GalCer was administered or not. However, where α-GalCer was used, the cytotoxic response was markedly greater, a likely reflection of the overall increase in the specific CTL pool in these animals.

As a further assessment of functional activity, peptide-specifc ELISPOT assays were used to assess IFNγ production from the induced $OVA_{257-264}$ $CD8^+$ T cell population. In addition, ELISPOT assays were also used to determine whether responses to the MHC class II binding peptides $OVA_{323-339}$ and $OVA_{265-280}$ were similarly affected by α-GalCer treatment. For these analyses splenocytes were taken from animals 11 days after antigen administration and stimulated with the specific peptides in vitro for 48 h (Fig. 1C). IFNγ responses to the class I peptide, $OVA_{257-264}$, were in agreement with the MHC class I tetramer data, with co-administration of α-GalCer and

ovalbumin leading to increased numbers of specific IFNγ-producing cells over levels found in animals treated with ovalbumin and vehicle (3-fold increase). This same trend was observed for both of the MHC class II binding peptides tested. In fact, very similar numbers of IFNγ-producing cells were seen for each of the treatment groups, regardless of peptide used. Thus, $CD4^+$ T cell responses are also increased by α-GalCer treatment.

As a final assessment of functional activity, the ability of the induced ovalbumin-specific T cells to respond to further stimulation in vivo was examined. Animals that had been treated with ovalbumin ± α-GalCer, or naïve controls, were therefore challenged with either $OVA_{257-264}$-loaded DC, or vaccinia encoding the whole chicken ovalbumin DNA sequence (Fig. 2). In both instances, $OVA_{257-264}$-specific populations were greatly expanded one week after restimulation in vivo. The responses were proportional to the initial responses following protein administration, so that in both assays the groups initially treated with ovalbumin and α-GalCer produced the most significant restimulatory responses. $OVA_{257-264}$-specific $CD8^+$ T cells induced in the absence of α-GalCer could also be restimulated, but the smaller populations induced likely reflect the initially smaller populations induced in the absence of α-GalCer.

These results show that administration of α-GalCer greatly enhanced take-up and presentation of blood borne ovalbumin protein to $CD4^+$ cells and to $CD8^+$ T cells.

NKT cells are required for the enhancing effect of α-GalCer on T cell responses induced with soluble antigen

While it has been clearly established that α-GalCer is a CD1d-binding ligand of NKT cells, it was still possible that the increased immune responses to i.v. injection of ovalbumin protein seen in the presence of α-GalCer was not directly

related to stimulation of activity from CD1d/α-GalCer-reactive NKT cells. For example, it was possible that increased T cell responses may result from interaction between ovalbumin protein and the glycolipid leading to efficient absorption of antigen by resident APC, perhaps via CD1d molecules. Therefore, to provide further proof that NKT cells were required for the α-GalCer-mediated increase in presentation to T cells (including cross presentation to $CD8^+$ T cells), immune responses to ovalbumin in animals that lack the Jα281 gene segment and consequently do not harbor NKT cells that can react to α-GalCer (27) were examined. As shown in Fig. 3A, co-administration of ovalbumin and α-GalCer to these $NKT^{-/-}$ animals did not lead to the increased activities seen in the wild-type animals as assessed by monitoring levels of $OVA_{257-264}$ $CD8^+$ T cells responses with tetramer in the blood on day 7 post ovalbumin injection, and by IFNγ ELISPOT assays on splenocytes taken on day 10. As a further assessment of the involvement of NKT cell activation in the enhancement of T cell responses to blood-borne protein, ovalbumin was injected into CD1d-deficient mice unable to present the α-GalCer glycolipid to NKT cells. Again, co-administration α-GalCer into $CD1d^{-/-}$ animals was not able to enhance ovalbumin-induced T cell responses (Fig. 3B). Finally, to examine the possibility that the glycolipid itself was directly enhancing antigen uptake of ovalbumin protein, ovalbumin was injected into wild-type animals together with α-GalCer, a glycolipid with similar structure to α-GalCer shown to bind to CD1d molecules, but without capacity to induce NKT cell activation (36). Co-administration of α-GalCer and protein did not lead to the dramatically enhanced levels of specific T cell responses that had been observed with α-GalCer. This shows that the enhanced responses observed with α-GalCer can be directly attributed to the activation of NKT cells.

The enhancing effect of NKT stimulation is dependent on timing of administration of α-GalCer relative to protein

The kinetics of NKT activation, cytokine release and subsequent activation induced cell death following α-GalCer injection have been well documented (26, 35, 37). Screens were performed to establish at which time-point(s) within these kinetics that the most significant enhancing effect of α-GalCer on immune responses to soluble antigen was observed. In the first screen, hallmark time-points for injection of α-GalCer relative to ovalbumin were chosen on the basis of the known serum cytokine profiles in response to α-GalCer. Thus, ovalbumin was injected 2 h after α-GalCer injection when IL-4 levels were known to be peaking in the serum, and 24 h after α-GalCer injection, when IFNγ levels were peaking. In both of these situations, α-GalCer administration had no enhancing effect on $OVA_{257-264}$ $CD8^+$ T cells responses measured by tetramer in the blood on day 7 post-ovalbumin injection (Fig. 4A). In addition, α-GalCer injected 24 h after ovalbumin did not enhance the ovalbumin-specific T cell responses. In fact, enhanced responses were only observed when the ovalbumin and α-GalCer were injected at the same time (Fig. 4A). However, a finer analysis of α-GalCer administration, between 2 h before and 8 h after ovalbumin administration, indicated that some enhancement could be observed if α-GalCer was administered within this period, with this effect diminishing 8 h after α-GalCer administration (Fig. 4B). Again, the most potent enhancement was observed when the ovalbumin and α-GalCer were injected at the same time. These data imply that enhancement of T cell responses may be dependent upon close temporal association between presentation of ovalbumin-derived peptides and α-GalCer molecules.

The enhancing effect of NKT cell stimunlation is dependent upon simultaneous presentation of α-GalCer and processed protein antigen by dendritic cells.

That NKT induced enhancement of T cell responses required close timing of α-GalCer and protein administration could reflect the need for simultaneous uptake of these molecules,

and perhaps a requirement for co-localization of their antigenic components on the same APC. In order to look at this possibility in more detail, immune responses to injection of cultured bone marrow-derived DC loaded with glycolipid and peptide antigens in vitro were examined. Intravenous injection of DC loaded with α-GalCer has been reported to induce cytokine release in vivo in much the same way as injection of free α-GalCer, although with some changes to the kinetics of release (38). Loading DC with α-GalCer in vitro did not induce maturation prior to injection, as determined by expression levels of CD80, CD86 and MHC class II (data not shown). Immune responses to the MHC class I-binding peptides LCMV GP_{34-41} (from lymphocytic choriomeningitis virus glycoprotein) (Fig. 5A) or $OVA_{257-264}$ (Fig. 5B) were assessed in the blood with MHC tetramers seven days after DC injection. For both of the MHC class I-binding peptides used, measurable immune responses were detected in animals injected with peptide loaded DC, as was expected. Importantly, in both experimental models, loading DC with peptide and α-GalCer resulted in reproducible 2-3 fold increases in immune responses (Fig. 5A + B). When immune responses to LCMV GP_{34-41} peptide were assessed in more detail, it was found that the α-GalCer-induced increase in immune response occurred only if the peptide and α-GalCer were presented on the same DC, rather than on separate DC (Fig. 5A, first panel). In addition, the enhancing effect of simultaneous presentation of peptide and α-GalCer was not seen when the DC were injected into $NKT^{-/-}$ hosts (Fig 5A, right panel), implying a direct role for the interaction of NKT with DC in producing this effect, presumably resulting in DC maturation. By maturing DC with LPS prior to injection, it was possible to enhance the immune response to LCMV GP_{34-41} peptide without addition of α-GalCer, with the level of enhancement similar to that seen with non-matured DC loaded with α-GalCer. However, when α-GalCer was loaded onto DC that had already been matured with LPS, no further increase in immune response was observed to LCMV GP_{34-41}. Together these data suggest that

the effect of loading DC with peptide and α-GalCer was to permit an interaction between DC and NKT cell that leads to DC maturation, and hence an improvement of the MHC class I-restricted immune response. This observation is very similar to the reported function of $CD4^+$ T cell help in the context of $CD8^+$ T cell activation (8-10). While the DC used in our experiments were cultured in FCS, and are therefore likely to be presenting some unique peptides to $CD4^+$ T cells in vivo, it would appear that this $CD4^+$ T cell help is insufficient to provide optimum DC maturation. We therefore examined whether addition of a known MHC class II-binding peptide would provide a more potent CD4-mediated maturation stimulus to DC, and whether α-GalCer-mediated NKT stimulation can fully substitute for this CD4 T cell help. The results, presented in Figure 5B, indicate that injection of DC loaded with both MHC class I binding peptide ($OVA_{257-264}$) and MHC class II binding peptide ($OVA_{323-339}$) resulted in a larger $OVA_{257-264}$-specific immune response than DC loaded with the class I binding peptide alone. However, this $CD4^+$ T cell-mediated help was not as significant as the response observed if the class II binding peptide is substituted with the addition of α-GalCer. Indeed, the α-GalCer-mediated enhanced response could not be improved even if α-GalCer was used together with both class I- and class II- binding peptides. Thus, it would appear that NKT are able to exert a 'helper' function that exceeds the potency of $CD4^+$ T cell mediated help. Finally, the effect of α-GalCer-mediated NKT activity was examined in the context of presentation of whole protein. DC were exposed to ovalbumin protein for 24 h before injection, a process that permitted some degree of antigen uptake and processing. A weak $OVA_{257-264}$-specific, response was observed in the blood seven days after DC injection, although the levels seen were statistically insignificant (Fig. 5C). However, if the ovalbumin-loaded DC were also loaded with α-GalCer, a significant immune response was observed. Loading with ovalbumin was done in the absence of NKT cells, so this result could not be attributed to an α-

GalCer-mediated NKT function that affects antigen uptake. It is therefore most likely that increased immune response reflects NKT cell-mediated maturation of DC in vivo. Again, as antigen processing should have provided both MHC class I- and class II- binding peptides in this experiment, the effect of NKT cell-mediated 'help' appears to be more potent than classical $CD4^+$ T cell-mediated help.

Injection of α-GalCer leads to maturation of splenic dendritic cells and increased cross presentation

To further investigate a role for NKT cell-mediated maturation of DC in vivo, the maturation status of DC taken directly from the spleens of animals injected with ovalbumin ± α-GalCer was examined. DC were isolated from the spleens of animals from both groups by positive selection using anti-CD11c coated magnetic beads 24 h after antigen injection. As a positive control on induction of DC maturation in vivo, a third group of animals was injected with lipopolysaccharide (LPS), a stimulus that has been shown to induce upregulation of co-stimulatory molecules on splenic DC in vivo (39). Maturation status was established by determining levels of expression of CD80 and CD86 on the isolated DC. Representative FACS profiles are shown in Fig. 6A. While injection of vehicle had no effect upon levels of CD80 and CD86 on DC relative to naïve controls, injection of α-GalCer induced an upregulation of expression of these co-stimulatory molecules that was equal to LPS, or in the case of CD80, even greater than LPS. These data suggest, therefore, that NKT cell activation provides a potent DC maturation stimulus.

The enhancing effect of NKT cell stimulation is CD40L dependent, but does not require INFγ

It has previously been reported that the provision of help by $CD4^+$ T cells is through interaction of T helper cells with DC, via CD40. CD40L is upregulated on $CD4^+$ T cells in response to T cell activation, and signals mediated by via CD40 on DC lead

to DC activation. NKT cells have also been reported to express CD40L upon activation (40) and it has therefore been suggested that NKT cells may also induce DC maturation and activation through interaction with CD40 (14, 16). To investigate the possibility that the enhanced ovalbumin-specific T cells responses induced with α-GalCer are CD40-mediated, ovalbumin protein was injected into $CD40L^{-/-}$ recipients together with α-GalCer or vehicle. $OVA_{257-264}$-specific $CD8^+$ T cell responses were monitored in the blood seven days later with tetramers. The α-GalCer-mediated enhancement of responses to ovalbumin administration was not observed in $CD40L^{-/-}$ recipients (Fig. 7A). Analysis of cytokine levels in serum in these recipients indicated that whereas α-GalCer induced both IFNγ and IL-4 release in wild-type animals, only IL-4 was induced in $CD40L^{-/-}$ recipients (Fig. 7B). Thus, while NKT cells were activated in $CD40L^{-/-}$ animals, they were unable to produce of IFNγ and enhance T cell responses in the absence of subsequent CD40/CD40L mediated signals.

In order to assess the possibility that IFNγ played a key role in NKT modulation of T cell responses, ovalbumin protein was injected into $IFN\gamma R^{-/-}$ recipients together with α-GalCer or vehicle (Fig 7C). Lack of a receptor for IFNγ made no difference to the induction of $OVA_{257-264}$-specific $CD8^+$ T cell responses measured in the blood, and importantly, did not affect the enhancement of this response by α-GalCer. Similarly, injection of ovalbumin protein together with OCH (Fig. 7D), an analogue of α-GalCer that induces activation of NKT cells with a profile of cytokine release predominated by IL-4 rather than IFNγ (30) (Fig. 7E), had no effect on NKT-mediated enhancement of T cell responses to the protein. These data suggest, therefore, that the enhancing effect of NKT cell stimulation on T cell responses does not require INFγ, but is CD40L dependent.

Oral administration of antigen together with alpha-GalCer induces functional CTL.

Murine iNKT cells are found with relative abundance in bone marrow, thymus, spleen and liver, while in low numbers in the blood and peripheral lymph nodes. It is likely, therefore, that this distribution of cells will determine the efficacy of administration of iNKT cell agonists by different routes. As vaccine administration via mucosal surfaces is generally seen as a preferred option in terms of likely patient uptake and ease of administration we examined the possibility of inducing immune responses by the oral route. Proteolytic activity in the digestive tract meant that the dose of OVA used was necessarily high, with 30 mg administered per animal. In many reported instances oral administration of protein at this dose has lead to the induction of T cell tolerance, rather than priming, highlighting a potential pitfall of administration of a vaccine by this route. Using tetramers to monitor immune responses in the blood 7 d after antigens were administered, small populations of $OVA_{257-264}$-specific T cells were detected in some animals treated with protein in vehicle (Figure 8a). Subsequent analysis of cytotoxic capacity of the induced populations in vivo by VITAL assay indicated insignificant cytotoxic function (Figure 8b), reflecting either the small size of the induced populations, or perhaps a blunted phenotype of the antigen-specific cells. In striking contrast, easily detected populations of $OVA_{257-264}$-specific cells, representing an average of 0.8% of $CD8^+$ cells, were induced when protein was administered orally together with alpha-GalCer. These populations had cytotoxic capacity, inducing up to 60% lysis of injected targets loaded with 5 μM $OVA_{257-264}$ peptideand could be restimulated in vivo by either vaccinia OVA or OVA in Freund adjuvant (data not shown). These data suggest that iNKT activation has a very powerful adjuvant effect capable of eliciting effective CTL by oral administration, a route generally considered unfavorable to stimulation of potent effector cells.

The enhancing effect of NKT cell stimulation is increased by stimulation of TLR4 on DC

The enhanced immune response observed after stimulating NKT cells can be significantly further enhanced by stimulating simultaneously the TLR4, which is expressed on DC. Stimulation of NKT cells, via either alphagal Cer or TCR stimulation leads to the maturation of DC upon the interaction of CD40L (expressed by activated NKT cells) and CD40 (expressed by DC). Stimulation of TLR4 (expressed by DC) has a synergistic effect with the CD40 dependent stimulation pathway and results in a much stronger immune response (Fig. 9). TLR4 binds LPS and a derivative of LPS "MPL", which is not toxic and can be injected into patients.

The experiment shown in Fig. 9 was carried out by incorporating alphaGalcer + ovalblumin + MPL into liposomes. The liposomes were injected i.v. into mice and 7 days later frequency of OVA specific CTL was measured by ex-vivo tetramer staining. Liposomes containing either ovalbumin and MPL or ovalbumin and alphaGalceramide were used a control. The results of these experiments show a very significant enhancement of the immune response in mice immunized with liposomes containing alphaGalcer + ovalblumin + MPL (i.e. OVA: 0.1% of tetramer+ cells; OVA + alphaGalcer: 2% of tetramer+ cells; OVA+alphaGalcer+MPL: 13% of tetramer+ cells.
We extended these results by demonstrating that co-injection of alphaGalceramide and MPL can mature DC more efficiently than either alphaGalceramide or MPL alone. Similar results were obtained using alphaGalceramide + the TLR 9 ligand Cpg, alphaGalceramide + the TLR5 ligand flagellin and alphaGalceramide + the TLR 3 ligand poly I:C (Fig. 10).

To assess whether the synergistic effect of alphaGalceramide + TLR ligands could be extended to other compounds capable of binding to CD1d molecules and activating NKT cells, experiments were carried out using the compound OCH (30),

which has a shorter sphyngosine chain as compared to alphaGalCer. Results of the experiments shown in Fig. 12 and 13 demonstrate that co-injection of OCH + MPL results in enhanced maturation of DC in vivo and enhanced frequency of OVA specific CTL.

Similar results were obtained in vitro demonstrating the ability of DC matured in vivo by OCH + MPL to stimulate in vitro proliferation and gamma secretion of splenocytes from mice transgenic for the $NP_{366-374}$ influenza NP peptide (Fig. 14).

Administration of α-GalCer together with soluble antigen provides potent anti-tumour responses

The therapeutic potential of initiating immune responses with soluble antigen and concomitant activation of NKT cells was investigated. The tumour cell line EG7.OVA, a derivative of the murine thymoma EL4 transfected with chicken ovalbumin cDNA (33), grows progressively when injected s.c. into C57BL/6 mice. When recipients were treated with ovalbumin together with vehicle 11 days prior to tumour challenge, no notable anti-tumour response was initiated, and tumour engraftment and progression was observed. This was despite a weak, but measurable $OVA_{257-264}$-specific $CD8^+$ T cell response in the blood at day 7 post ovalbumin injection (data not shown). In contrast, when the recipients were treated with ovalbumin together with α-GalCer prior to tumour challenge, significant resistance to tumour progression was initated, with the majority of animals remaining tumour free in excess of 40 days after tumour challenge. This experiment highlights the potential for immunotherapeutic strategies designed upon the knowledge that NKT stimulation can significantly potentiate T cell responses to soluble antigen.

Discussion

The ability of DC to promote or suppress adaptive responses to antigen may ultimately be defined by the signals received by

immature DC as they transit from peripheral sites to the draining lymph nodes, where adaptive responses are dictated. In this report we show that NKT cells responding to glycolipids presented by monomorphic CD1d molecules can significantly regulate T cell responses to soluble antigen in vivo, and that this modulation is through direct interaction with DC. NKT cells therefore represent an abundant source of the T cell derived factors required for DC activation, and that these cells can potentially be rapidly mobilized in the generation of T cell mediated responses. Crucially, NKT cells can provide an early source of CD40L signals that are known to be required for Th1-biased responses, and CTL induction.

Activation of NKT cells by administration of α-GalCer together with intravenous delivery of soluble ovalbumin protein results in considerable enhancement of ovalbumin-specific T cell responses, including CTL responses reliant upon cross presentation. Analysis of splenic DC isolated from animals injected with α-GalCer indicated that NKT activation induces upregulated expression of co-stimulatory molecules reflecting a more mature DC status. Thus, appropriately timed administration of protein relative to α-GalCer allowed processed protein to be presented on DC with enhanced stimulatory function. A cognate interaction between NKT cell and DC was critical to the enhancement of T cell responses observed as the potentiating effect of α-GalCer on injection of peptide-loaded BM-derived DC occurred only when the glycolipid was presented on the same DC as the peptide. These experiments were undertaken with DC that had been loaded with a minimal MHC class I-binding peptide, rather than whole protein, so that $CD8^+$ T cell responses could be interpreted without any potentially confounding effects of NKT cells on protein processing and presentation.

The effect of NKT interaction on DC function proved to be a more powerful stimulus to $CD8^+$ T cell responses than induction

of conventional $CD4^+$ T cell help provided by loading both MHC class II-binding and NHC class I-binding peptides onto the injected DC. This is presumably due to the greater number of NKT cells in the lymphoid tissues relative to naïve antigen-specific $CD4^+$ T cells, and hence an improved probability of interacting with injected DC. DC matured with LPS prior to injection also enhanced the peptide-specific $CD8^+$ T cell responses over those induced with non-matured DC, although this enhancement could not be improved upon with NKT stimulation using α-GalCer. However, the enhancement of T-cell response seen with α-GalCer in vivo was increased by the LPS derivative MPL.

Efficient cross priming of $CD8^+$ T cells requires uptake of antigen by immature DC together with exposure to effective maturation stimuli. The most important stimuli in this regard are thought to be CD40L signals, generally presumed to come from $CD4^+$ T helper cells (8-10). Schulz et al. (41) have reported that effective CD40 triggering of DC, as measured by IL-12 induction, requires initial microbial priming of the DC. In our system, where intravenous injection of soluble ovalbumin alone induced cross-priming, it is most likely that low levels of endotoxin in the commercial source of ovalbumin used was providing such a microbial signal. This signal could then combine with conventional $CD4^+$ T cell-mediated help to induce priming of $CD8^+$ responses. The fact that all cross priming was abrogated in $CD40L^{-/-}$ animals highlights the importance of integrating microbial and CD40-mediated signals in this system. Importantly, however, cross-priming in CD40 proficient animals was always significantly enhanced when α-GalCer was administered together with ovalbumin, most likely reflecting a greater supply of CD40L signals from stimulated NKT cells. Thus, while functional CTL with restimulatory capacity could be induced with ovalbumin alone, a dramatic increase in the quantity of CTL (5-10 times in number) was observed when NKT were concomitantly activated with α-GalCer.

Furthermore, only CTL induced in the presence of α-GalCer provided resistance to challenge with ovalbumin$^+$ tumours. Thus, activated NKT cells can provide a potent CD40L-mediated boost to an otherwise weakly cross-presented response, providing functionally superior CTL.

It is likely that a number of reciprocal exchanges between NKT cell and DC will ultimately determine the quality of an adaptive response. Initial engagement between NKT and APC bearing glycolipid ligand leads to release of a burst of IL-4 by NKT cells, which can be achieved in the absence of CD40 signalling ((42) and Fig. 7). This initial engagement may then lead to increased expression of CD40L on the NKT cell surface and subsequent interaction with DC expressing CD40. As a consequence, DC will be induced to release IL-12, which, in turn, stimulates NKT cells to release IFNγ. It has been suggested that IFNγ produced by NKT cells can induce upregulation of IL-12 receptors on NKT cells in an autocrine manner (14). These cells therefore become more sensitive to IL-12, resulting in further IFNγ production. It is also possible that IFNγ can, in turn, feed back on the DC causing more release of IL-12, as has been reported for human DC (43). Other studies have highlighted the events subsequent from this point, namely the activation of NK cells and release of further IFNγ (44). The inventors demonstrate that a second outcome of this interaction in which the CD40-mediated activation of DC leads to upregulation of T cell stimulatory machinery, including co-stimulatory molecules, and a greatly enhanced capacity to stimulate naïve T cells reactive to foreign antigen.

If this cycle of events is blocked as a result of inability to stimulate through CD40, as was the case in CD40L$^{-/-}$ recipients, NKT cells could still respond to α-GalCer by producing IL-4 (Fig. 7B), but the activation of DC could not take place. On the other hand, the induction of IFNγ1, an event that occurs

subsequent to CD40 signalling, is not crucial to the enhancement of T cell responses to soluble antigen, as potentiation of $CD8^+$ T cell responses was still observed in $IFN\gamma R^{-/-}$ mice (Fig. 7C) and also when NKT were stimulated with an analogue of α-GalCer with reduced capacity to induce IFNγ (Fig. 7D). These data imply that it is the cognate interaction between NKT cells and DC that is dictating enhancement of T cell responses to soluble antigen, and not a potentiating effect of IFNγ on DC function.

Studies have shown that α-GalCer can stimulate human Vα24/Vβ11 cells restricted by CD1d. Activated Vα24/Vβ11 NKT cells can exert cytotoxic activity against a number of human tumours in vitro, regardless of histological origin (46). Animal studies have also highlighted the utility of this glycolipid in the induction of anti-tumour responses. However, these latter studies have suggested that direct NKT cell-mediated cytoxixicty does not play a significant role in responses to tumours in vivo (47). In fact it is the NKT cell-mediated activation of NK cells, and rapid induction of IFNγ, that exerts the anti-tumour activity (48), with the anti-angiogenic property of IFNγ playing a key role (49). It has been reported that some anti-tumour CTL activity may also be invoked through the NKT activation with α-GalCer (50, 51). This activity may be dependent upon NKT-mediated activation of DC that have acquired unique tumour antigens. While this may happen to a limited degree in tumour-bearing hosts treated with α-GalCer alone, a more successful anti-tumour response would be induced if concomitant presentation of tumour antigen and α-GalCer was coordinated by injection of unique tumour proteins with the glycolipid. In the experiments described herein, resistance to challenge with ovalbumin$^+$ tumours was induced in such a fashion (Fig. 9). Therapy of this type could potentially be used in all cancer patients, regardless of MHC haplotype, as the adjuvant capacity of NKT cells is dependent upon presentation of α-GalCer by monomorphic CD1d molecules.

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CLAIMS:

1. Use of an activator of NKT cells in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with a TLR activator, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

2. Use of a TLR activator in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with an activator of NKT cells, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

3. Use according to claim 1 or claim 2 wherein the activator of NKT cells is α-GalCer.

4. Use according to claim 1 or claim 2 wherein the activator of NKT cells is OCH.

5. Use according to any one of claims 1 to 4, wherein the TLR is TLR3, TLR4, TLR5, TLR7 or TLR9.

6. Use according to claim 5, wherein the activator of TLR4 is MPL.

7. Use according to any one of claims 1 to 6, wherein the activator of NKT cells is administered up to about 2 hours before the polypeptide antigen is administered to said individual.

8. Use according to any one of claims 1 to 6, wherein the activator of NKT cells is administered up to about 8 hours

after the polypeptide antigen is administered to said individual.

9. Use according to any one of claims 1 to 6, wherein the activator of NKT cells and the polypeptide antigen are administered concurrently to said individual.

10. Use according to any one of claims 1 to 9, wherein the soluble antigen is a tumour antigen.

11. Use according to any one of claims 1 to 9 wherein the soluble antigen is a viral antigen.

12. Use according to any one of claims 1 to 9, wherein the soluble antigen is a bacterial antigen.

13. Use of OCH in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual.

14. Use according to claim 13, wherein the OCH is administered up to about 2 hours before the polypeptide antigen is administered to said individual.

15. Use according to claim 13, wherein the OCH is administered up to about 8 hours after the polypeptide antigen is administered to said individual.

16. Use according to claims 13, wherein the OCH and the polypeptide antigen are administered concurrently to said individual.

17. Use according to any one of claims 13 to 16, wherein the soluble antigen is a tumour antigen.

18. Use according to any one of claims 13 to 16 wherein the soluble antigen is a viral antigen.

19. Use according to any one of claims 13 to 16, wherein the soluble antigen is a bacterial antigen.

20. Use of α-GalCer in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with CpG or MPL.

21. Use of α-glucosylceramide in the preparation of a medicament for inducing an immune response to soluble polypeptide antigen in an individual, wherein the medicament is for administration in conjunction with CpG or MPL.

22. A composition comprising an activator of NKT cells and a TLR activator, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

23. A composition according to claim 22 wherein the activator of NKT cells is α-GalCer.

24. A composition according to claim 22 wherein the activator of NKT cells is OCH.

25. A composition according to any one of claims 22 to 24, wherein the TLR is TLR3, TLR4, TLR5, TLR7 or TLR9.

26. A composition according to claim 25, wherein the activator of TLR4 is MPL.

27. A composition comprising α-GalCer and MPL or CpG.

28. A composition comprising α-glucosylceramide and MPL or CpG.

29. A composition according to any one of claims 22 to 28 further comprising a purified soluble polypeptide antigen.

30. A composition comprising OCH and a purified soluble polypeptide antigen.

31. A composition according to claim 29 or claim 30, which composition is free of cells.

32. A composition according to any one of claims 29 to 31, wherein the soluble antigen is a tumour antigen.

33. A composition according to any one of claims 29 to 31, wherein the soluble antigen is a viral antigen.

34. A composition according to any one of claims 29 to 31, wherein the soluble antigen is a bacterial antigen.

35. A pharmaceutical composition comprising the composition of any of claims 22 to 34 and a pharmaceutically acceptable carrier or diluent.

36. The pharmaceutical composition of claim 35 wherein the carrier is a liposome.

37. Use of the composition of any of claims 22 to 34 or the pharmaceutical composition of claim 35 or claim 36 in a method of inducing an immune response in an individual.

38. A kit having first and second containers, wherein the first container comprises a composition comprising an activator of NKT cells, and the second container

comprises a composition comprising a TLR activator, with the proviso that, when the activator of NKT cells is a glycosylceramide, the TLR activator is not CpG or MPL.

39. A kit having first and second containers, wherein the first container comprises a composition comprising α-GalCer, and the second container comprises a composition comprising CpG or MPL.

40. A kit having first and second containers, wherein the first container comprises a composition comprising α-glucosylceramide, and the second container comprises a composition comprising CpG or MPL.

41. A kit according to any one of claims 38 to 40 which additionally has a third container, which container comprises a composition comprising a purified polypeptide antigen.

42. A kit having first and second containers, wherein the first container comprises a composition comprising OCH, and the second container comprises a composition comprising a purified polypeptide antigen.

43. A kit according to any one of claims 38 to 42 wherein any or all of said compositions further comprises a pharmaceutically acceptable carrier or diluent.

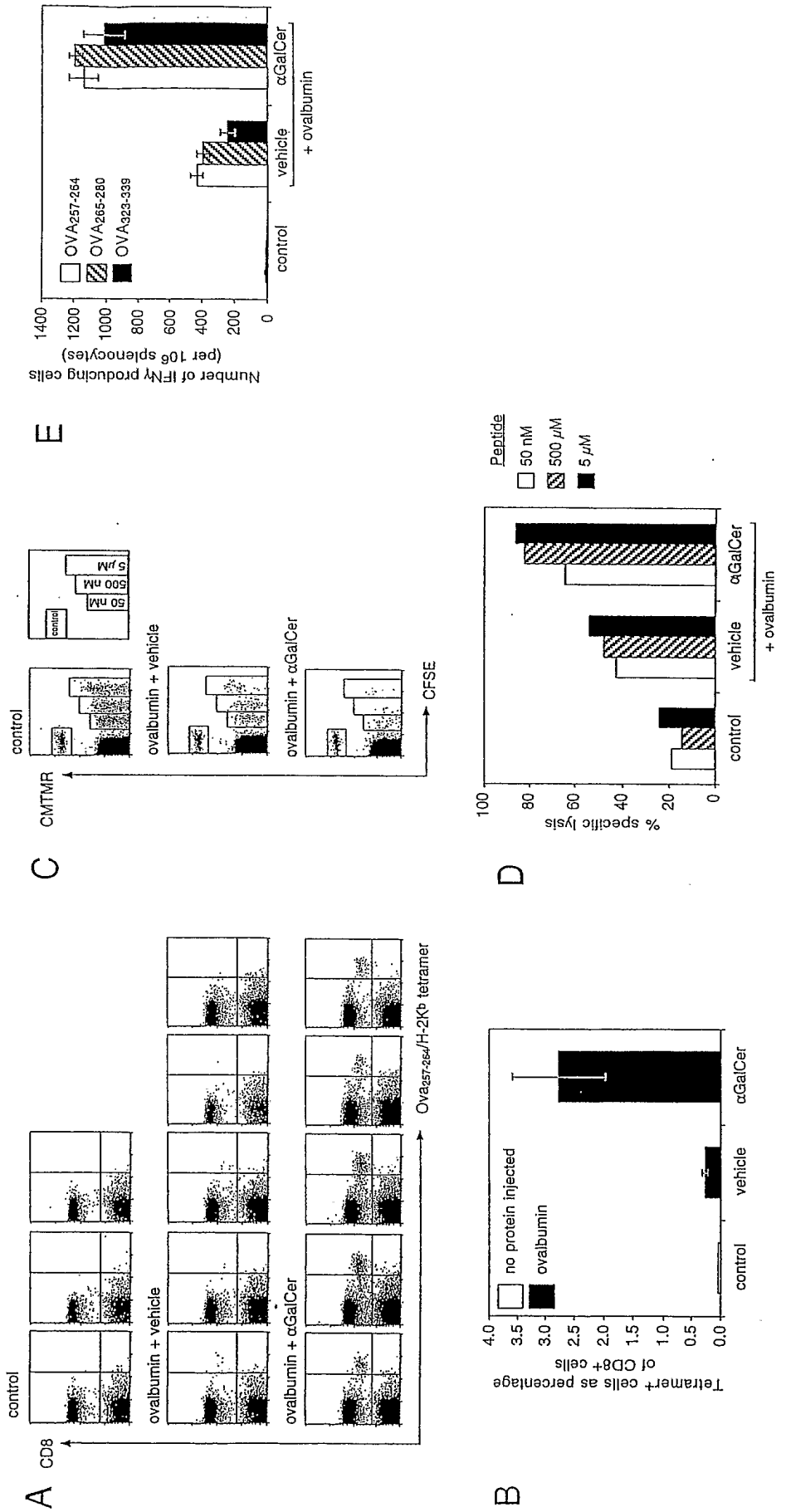

A
CD8
control
ovalbumin + vehicle
ovalbumin + αGalCer
Ova257-264/H-2Kb tetramer
B
Tetramer+ cells as percentage of CD8+ cells
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
no protein injected
ovalbumin
control
vehicle
αGalCer
C
CMTMR
control
ovalbumin + vehicle
ovalbumin + αGalCer
CFSE
control
50 nM
500 nM
5 μM
D
% specific lysis
100
80
60
40
20
0
Peptide
50 nM
500 μM
5 μM
control
vehicle
αGalCer
+ ovalbumin
E
Number of IFNγ producing cells (per 10⁶ splenocytes)
1400
1200
1000
800
600
400
200
0
OVA257-264
OVA265-280
OVA323-339
control
vehicle
αGalCer
+ ovalbumin

Figure 1

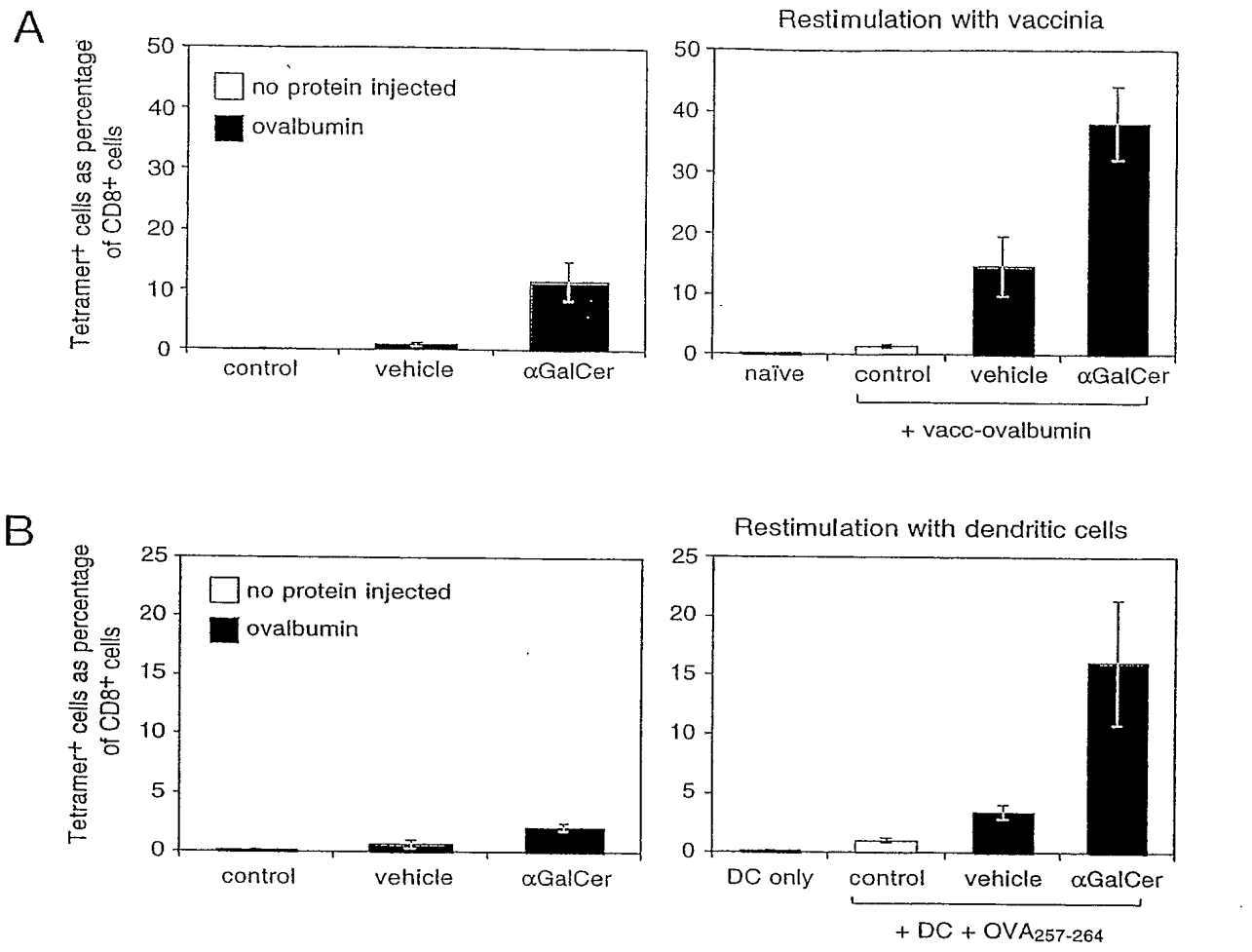

A
Tetramer+ cells as percentage of CD8+ cells
no protein injected
ovalbumin
control
vehicle
αGalCer
Restimulation with vaccinia
naïve
control
vehicle
αGalCer
+ vacc-ovalbumin
B
Tetramer+ cells as percentage of CD8+ cells
no protein injected
ovalbumin
control
vehicle
αGalCer
Restimulation with dendritic cells
DC only
control
vehicle
αGalCer
+ DC + OVA257-264

Figure 2

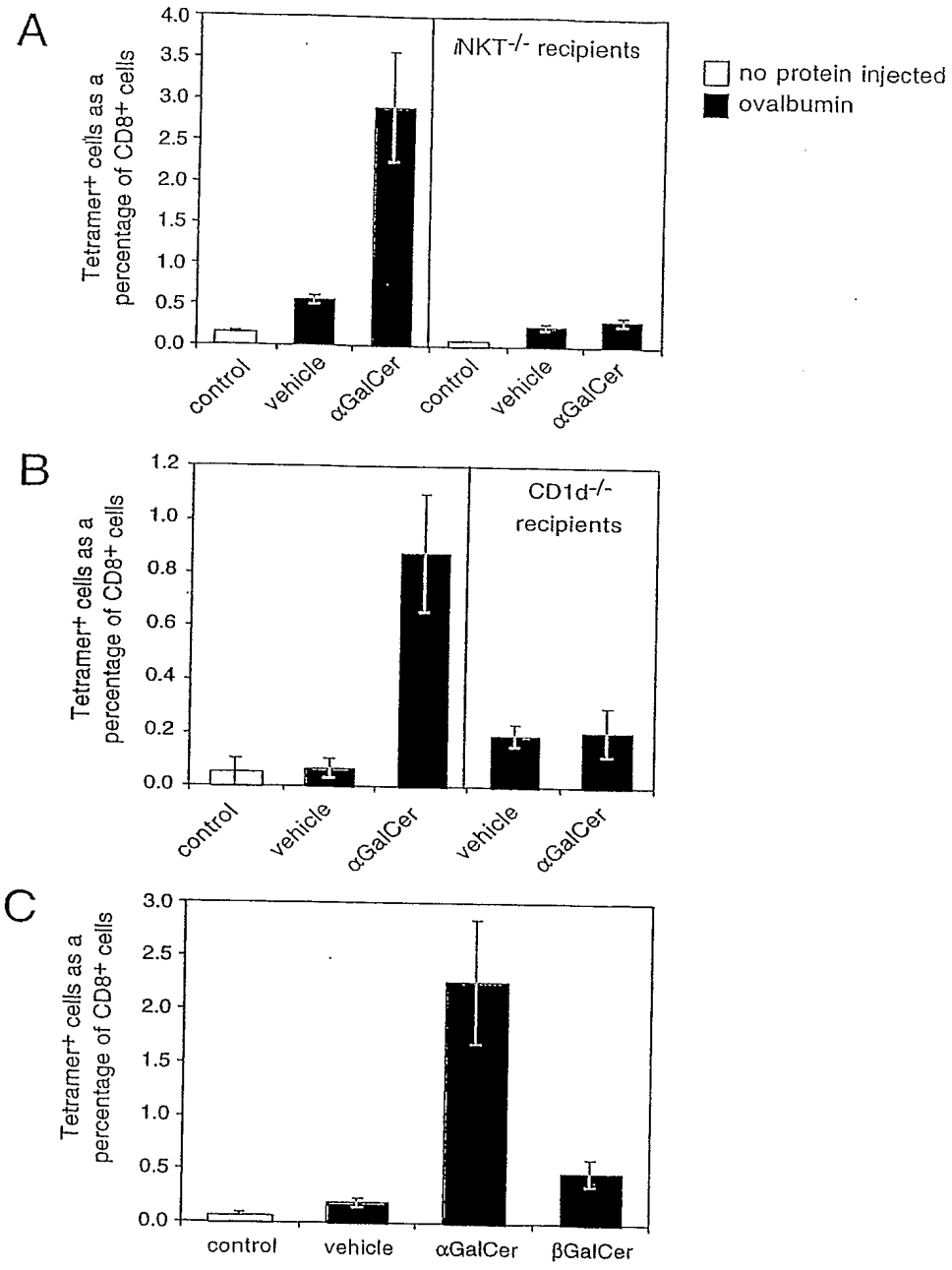

A
Tetramer+ cells as a percentage of CD8+ cells
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
iNKT-/- recipients
no protein injected
ovalbumin
control
vehicle
αGalCer
control
vehicle
αGalCer
B
Tetramer+ cells as a percentage of CD8+ cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CD1d-/- recipients
control
vehicle
αGalCer
vehicle
αGalCer
C
Tetramer+ cells as a percentage of CD8+ cells
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
vehicle
αGalCer
βGalCer

Figure 3

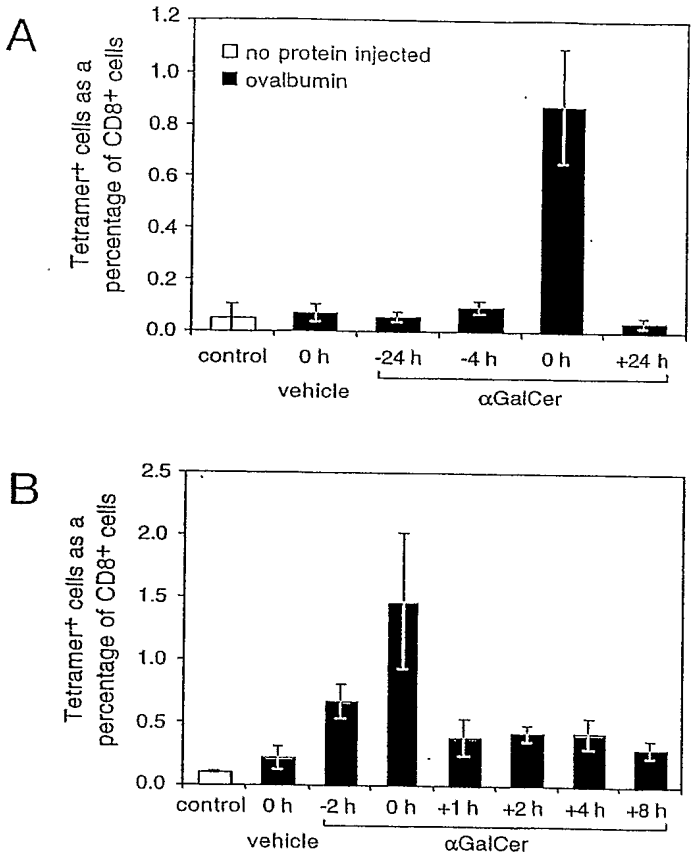
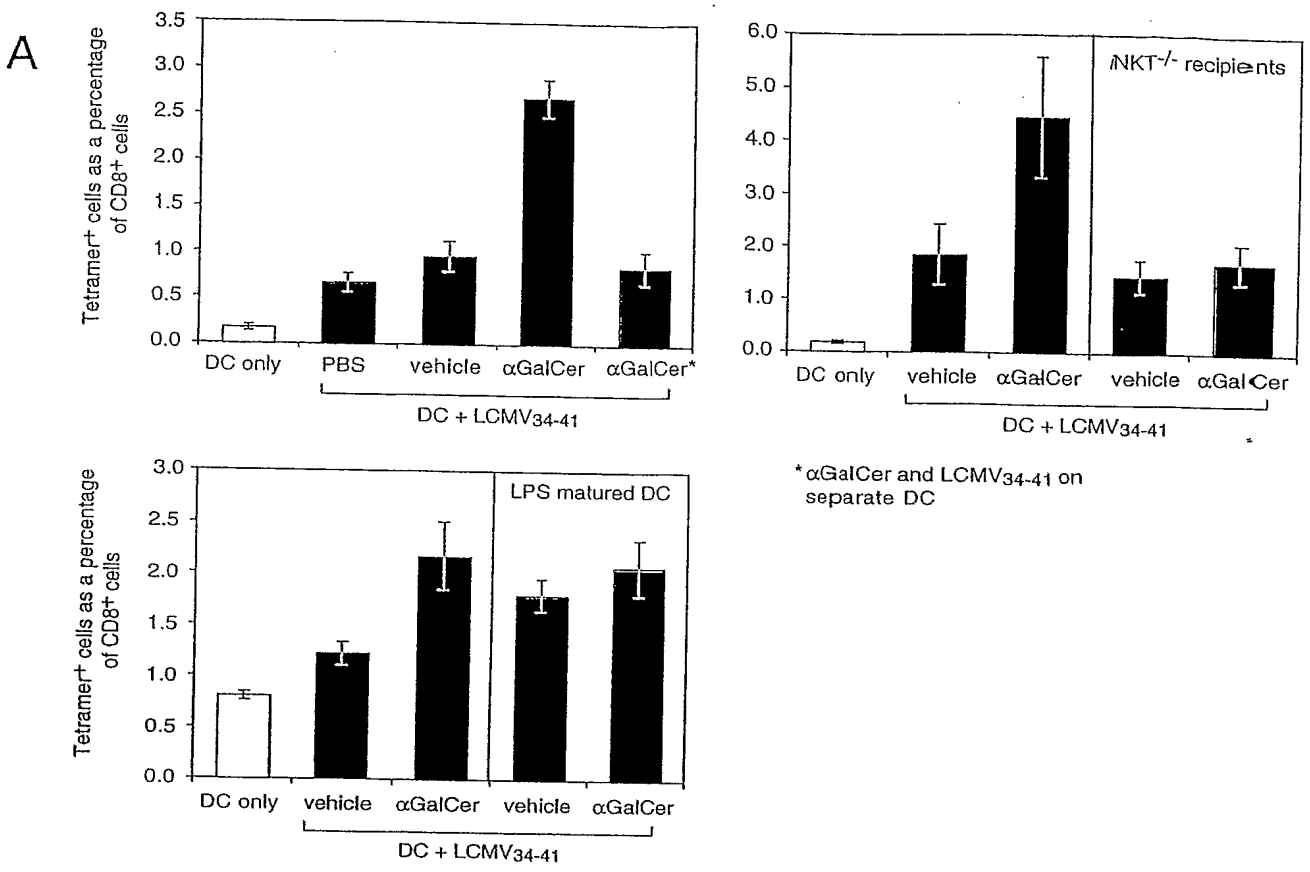
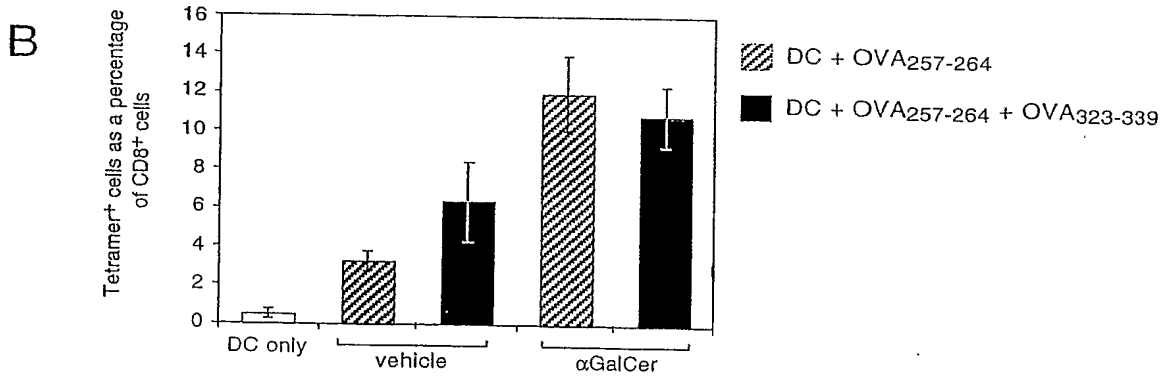

A
Tetramer+ cells as a percentage of CD8+ cells
no protein injected
ovalbumin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
control
0 h
-24 h
-4 h
0 h
+24 h
vehicle
αGalCer
B
Tetramer+ cells as a percentage of CD8+ cells
2.5
2.0
1.5
1.0
0.5
0.0
control
0 h
-2 h
0 h
+1 h
+2 h
+4 h
+8 h
vehicle
αGalCer

Figure 4


A
Tetramer+ cells as a percentage of CD8+ cells
DC only
PBS
vehicle
αGalCer
αGalCer*
DC + LCMV34-41
iNKT-/- recipients
*αGalCer and LCMV34-41 on separate DC
LPS matured DC


B
Tetramer+ cells as a percentage of CD8+ cells
DC only
vehicle
αGalCer
DC + OVA257-264
DC + OVA257-264 + OVA323-339

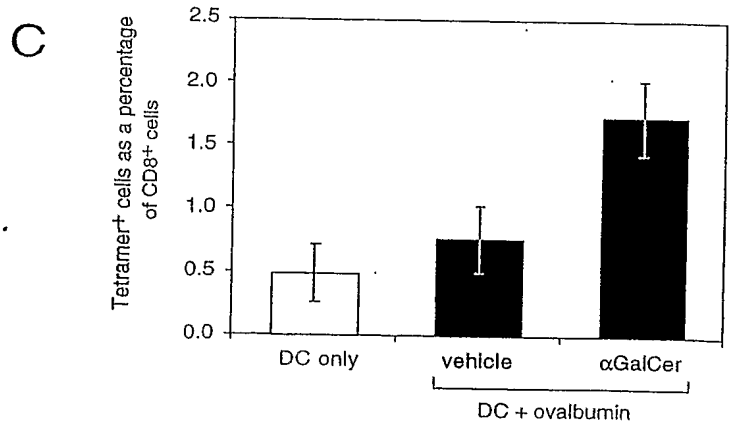

C
Tetramer+ cells as a percentage of CD8+ cells
DC only
vehicle
αGalCer
DC + ovalbumin

Figure 5

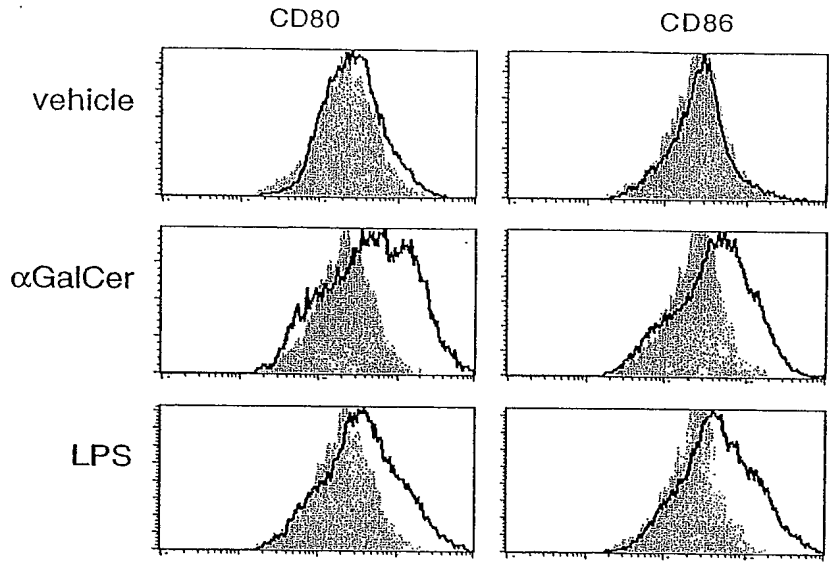


Figure 6

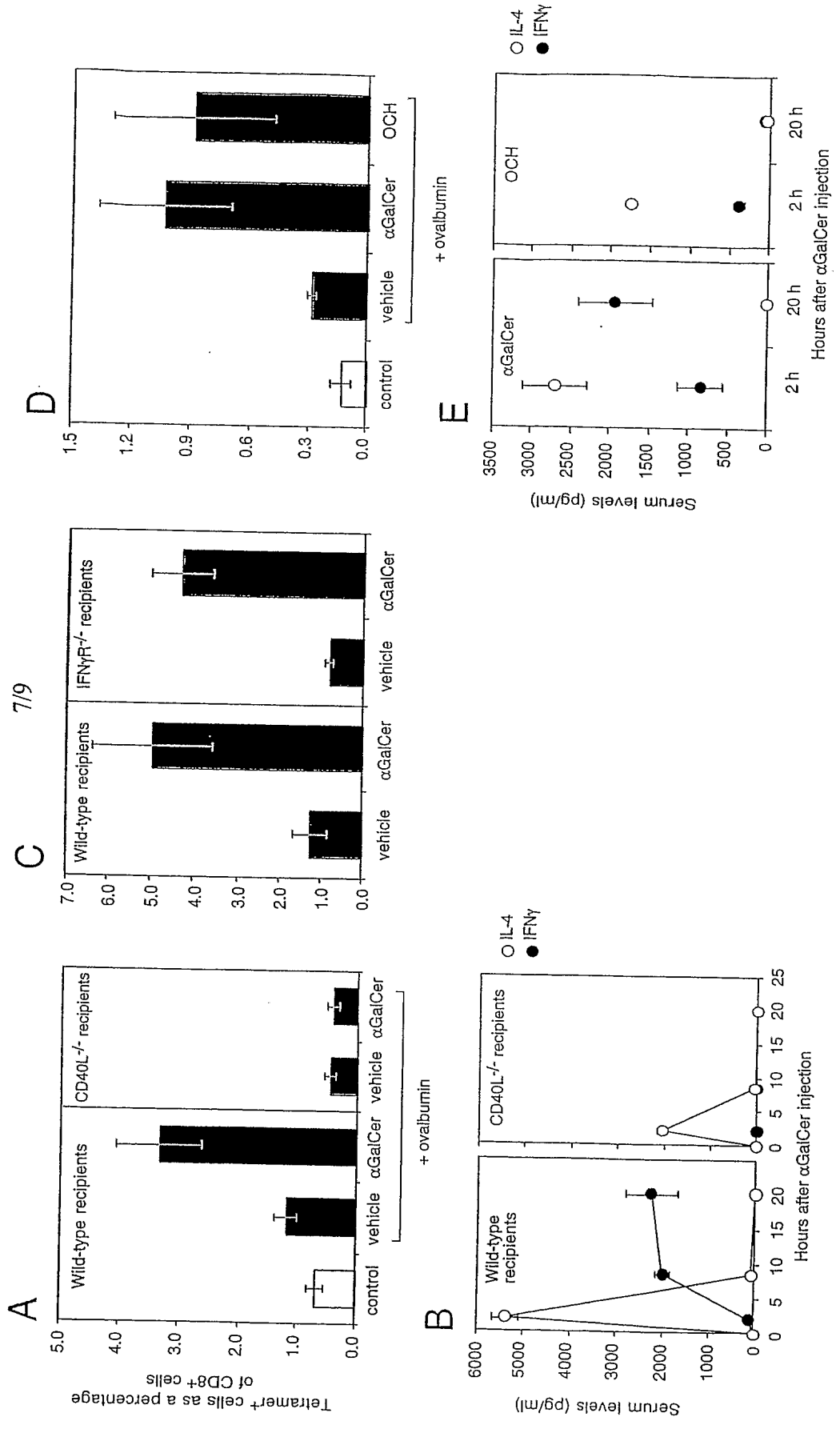

A
Wild-type recipients
CD40L-/- recipients
Tetramer+ cells as a percentage of CD8+ cells
control
vehicle
αGalCer
+ ovalbumin
B
Wild-type recipients
CD40L-/- recipients
IL-4
IFNγ
Serum levels (pg/ml)
Hours after αGalCer injection
C
Wild-type recipients
IFNγR-/- recipients
D
control
vehicle
αGalCer
OCH
+ ovalbumin
E
αGalCer
OCH
2 h
20 h
Serum levels (pg/ml)
Hours after αGalCer injection

Figure 7

Figure 8(a)

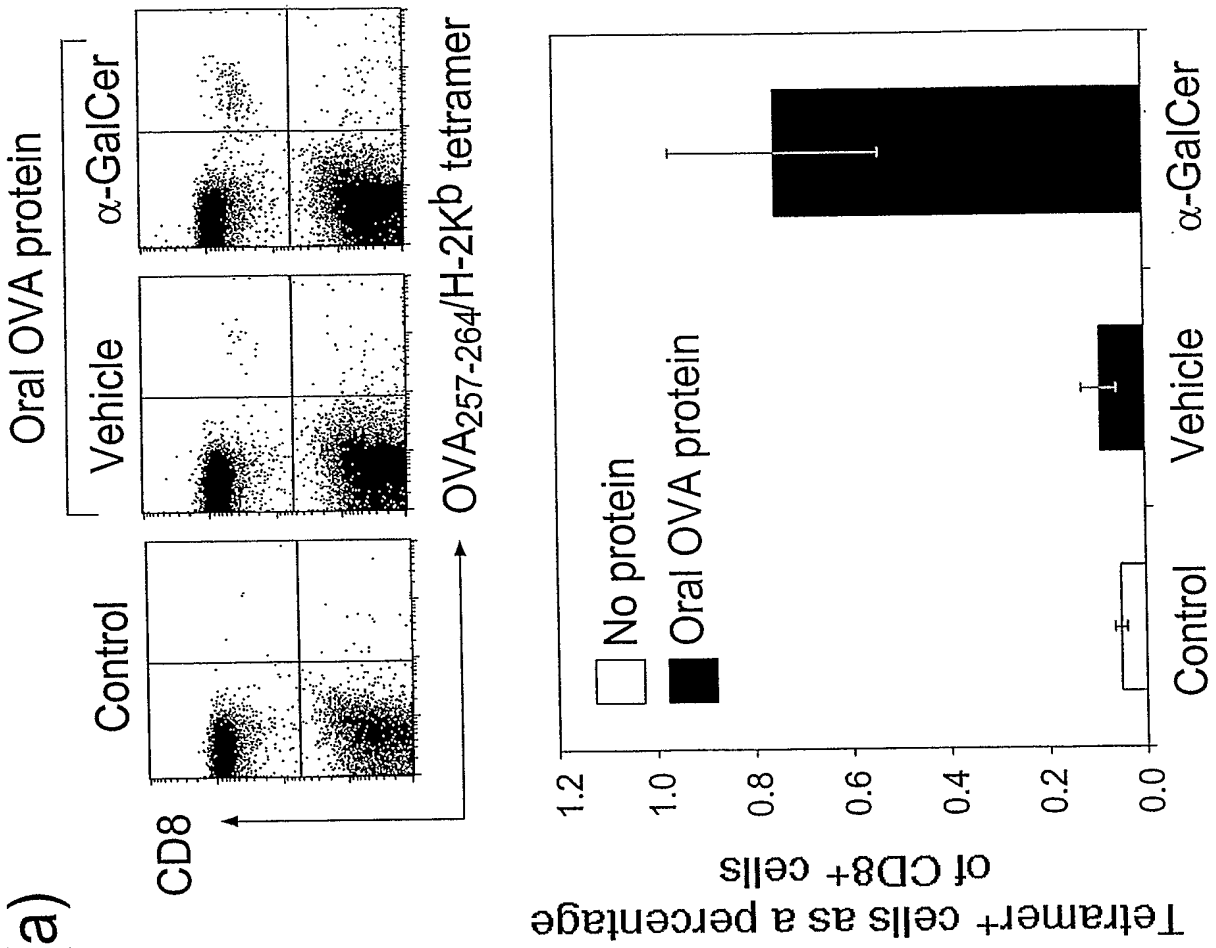
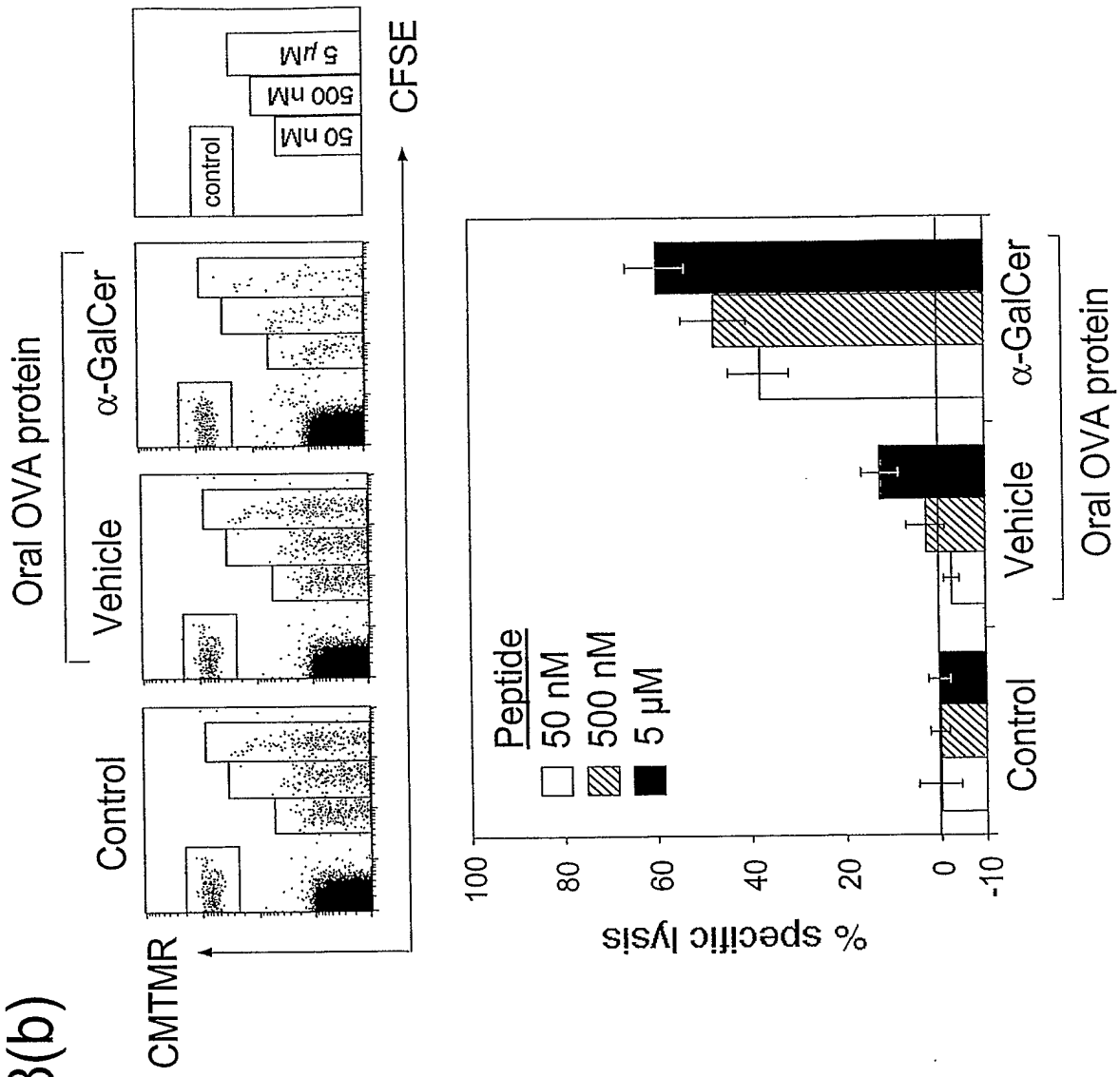

Oral OVA protein
Control
Vehicle
α-GalCer
CD8
OVA257-264/H-2Kb tetramer
No protein
Oral OVA protein
Tetramer+ cells as a percentage of CD8+ cells
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Vehicle
α-GalCer

Figure 8(b)


Oral OVA protein
Vehicle
α-GalCer
Control
CMTMR
CFSE
control
50 nM
500 nM
5 μM
Peptide
50 nM
500 nM
5 μM
% specific lysis
100
80
60
40
20
0
-10
Control
Vehicle
α-GalCer
Oral OVA protein

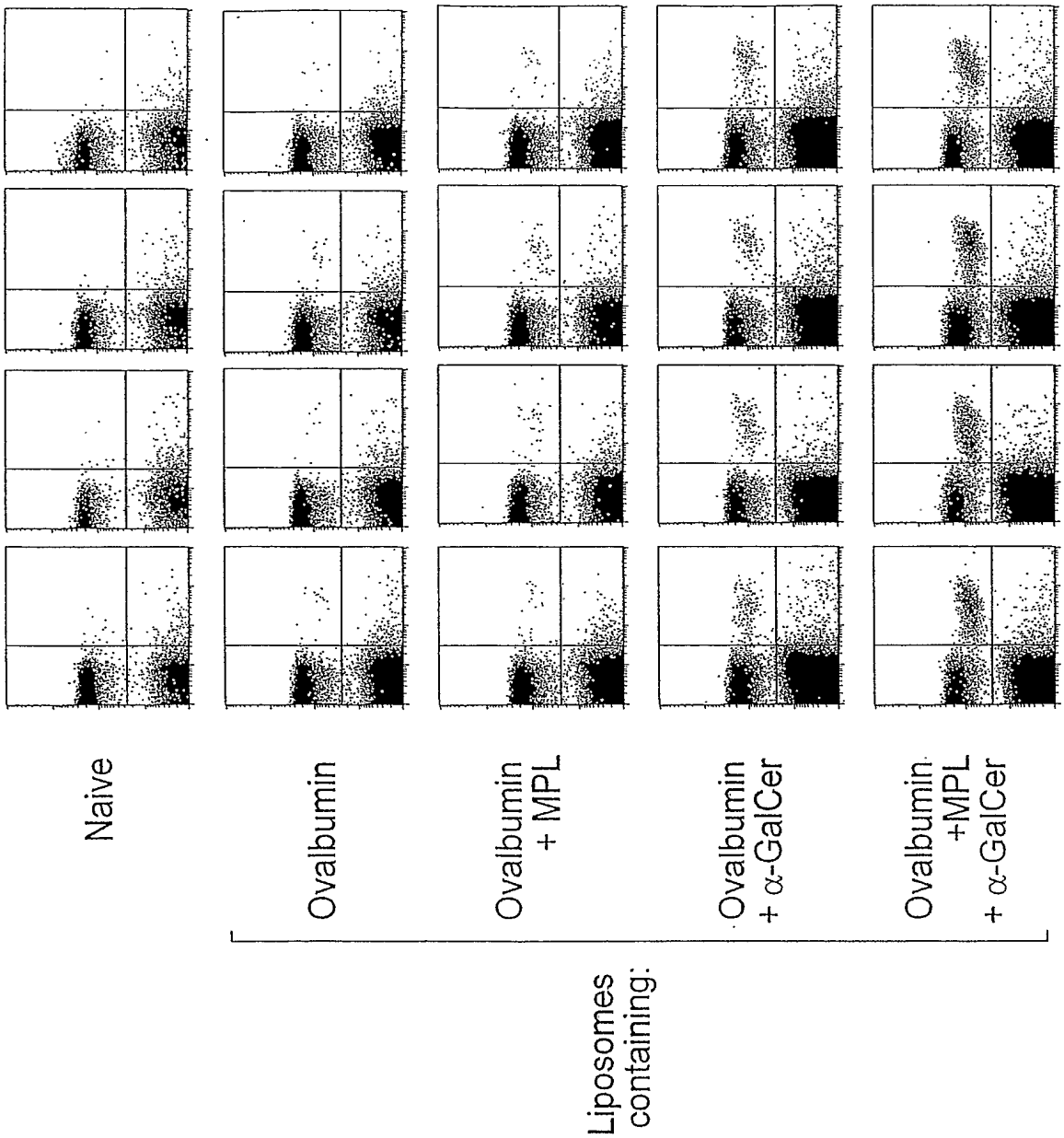
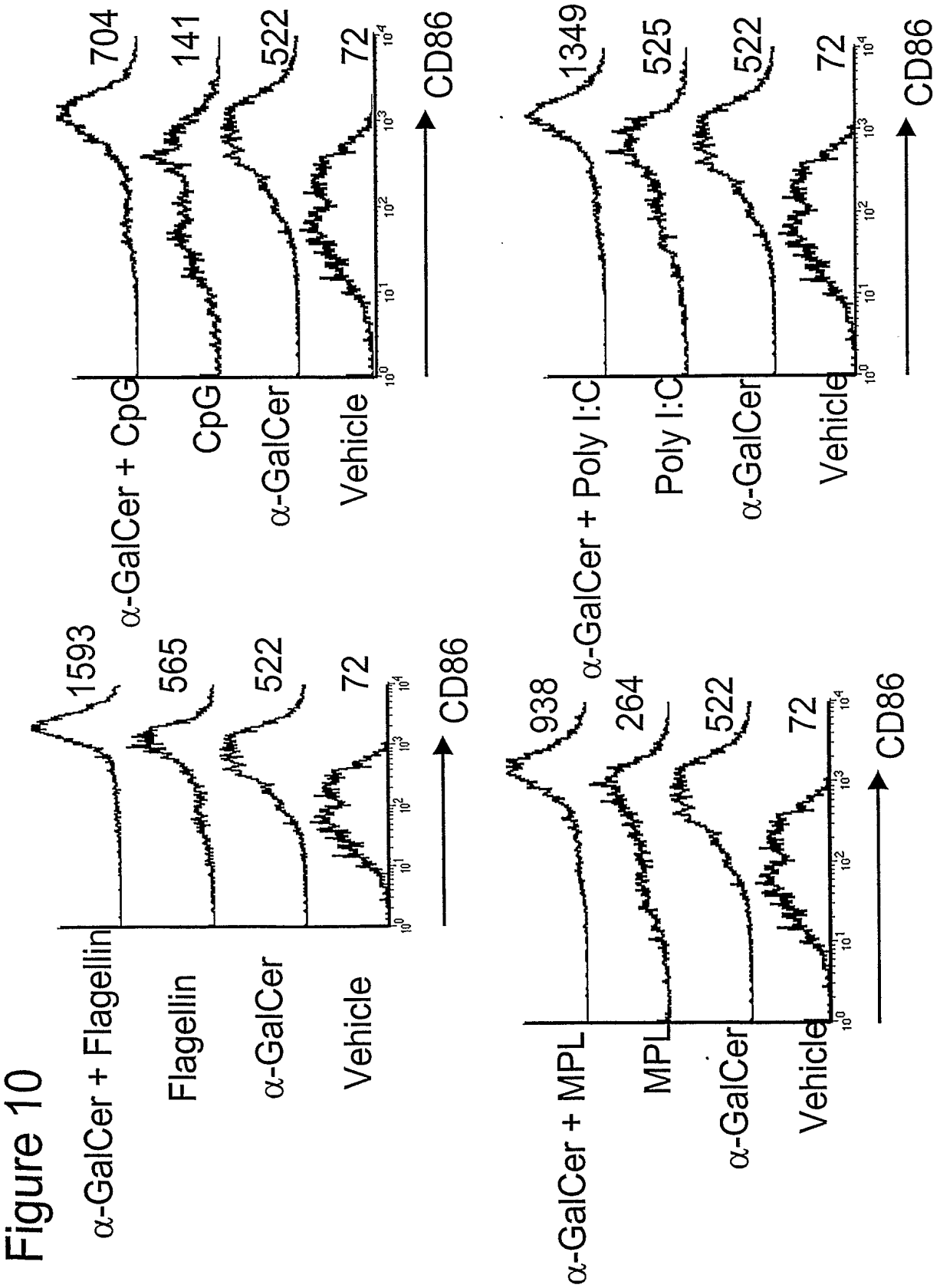

Naive
Ovalbumin
Ovalbumin + MPL
Ovalbumin + α-GalCer
Ovalbumin +MPL + α-GalCer
Liposomes containing:

Figure 9

Figure 10


α-GalCer + Flagellin
Flagellin
α-GalCer
Vehicle
1593
565
522
72
CD86
α-GalCer + CpG
CpG
α-GalCer
Vehicle
704
141
522
72
CD86
α-GalCer + MPL
MPL
α-GalCer
Vehicle
938
264
522
72
CD86
α-GalCer + Poly I:C
Poly I:C
α-GalCer
Vehicle
1349
525
522
72
CD86

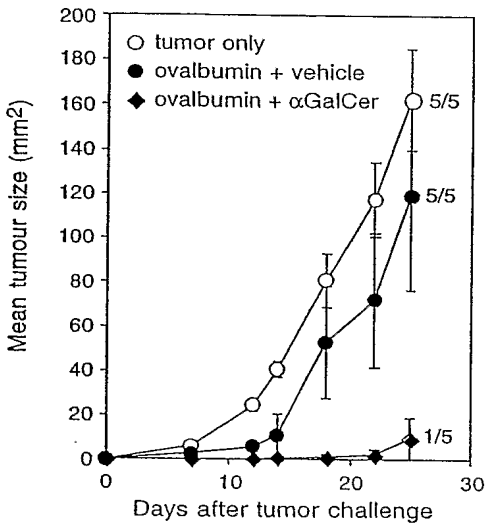

200
180
160
140
120
100
80
60
40
20
0
Mean tumour size (mm2)
tumor only
ovalbumin + vehicle
ovalbumin + αGalCer
5/5
5/5
1/5
0
10
20
30
Days after tumor challenge

Figure 11

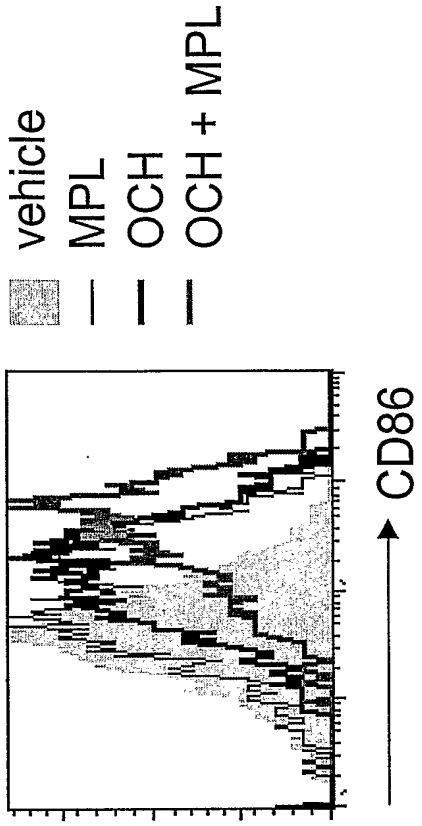


Figure 12

Figure 13(a)

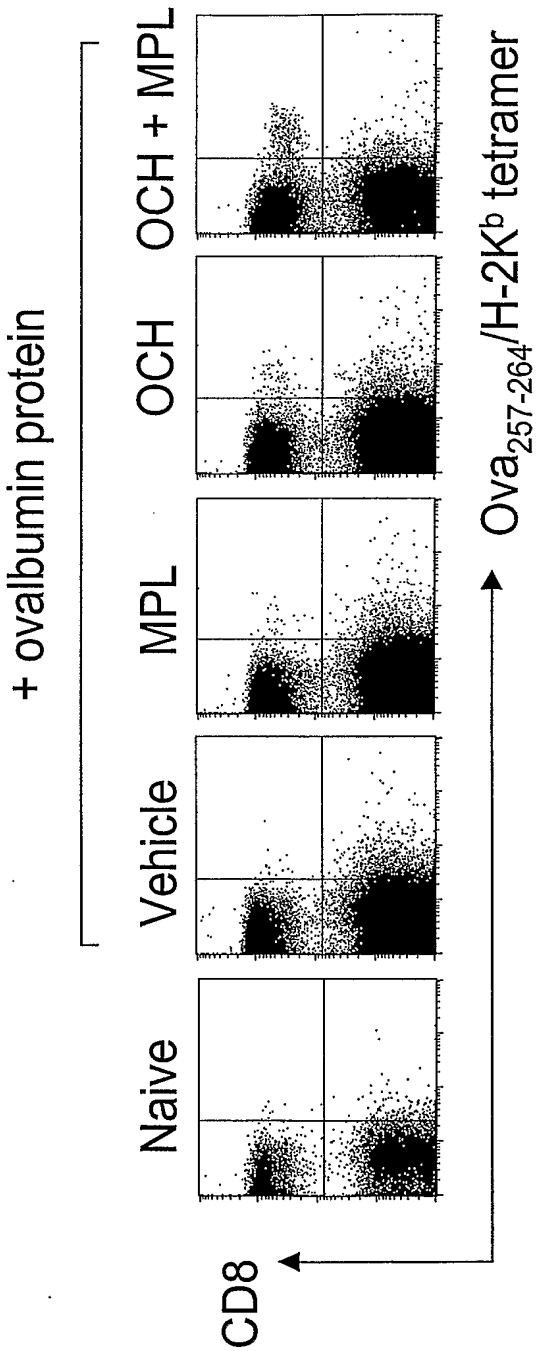

+ ovalbumin protein
Naive
Vehicle
MPL
OCH
OCH + MPL
CD8
Ova257-264/H-2Kb tetramer

Figure 13(b)

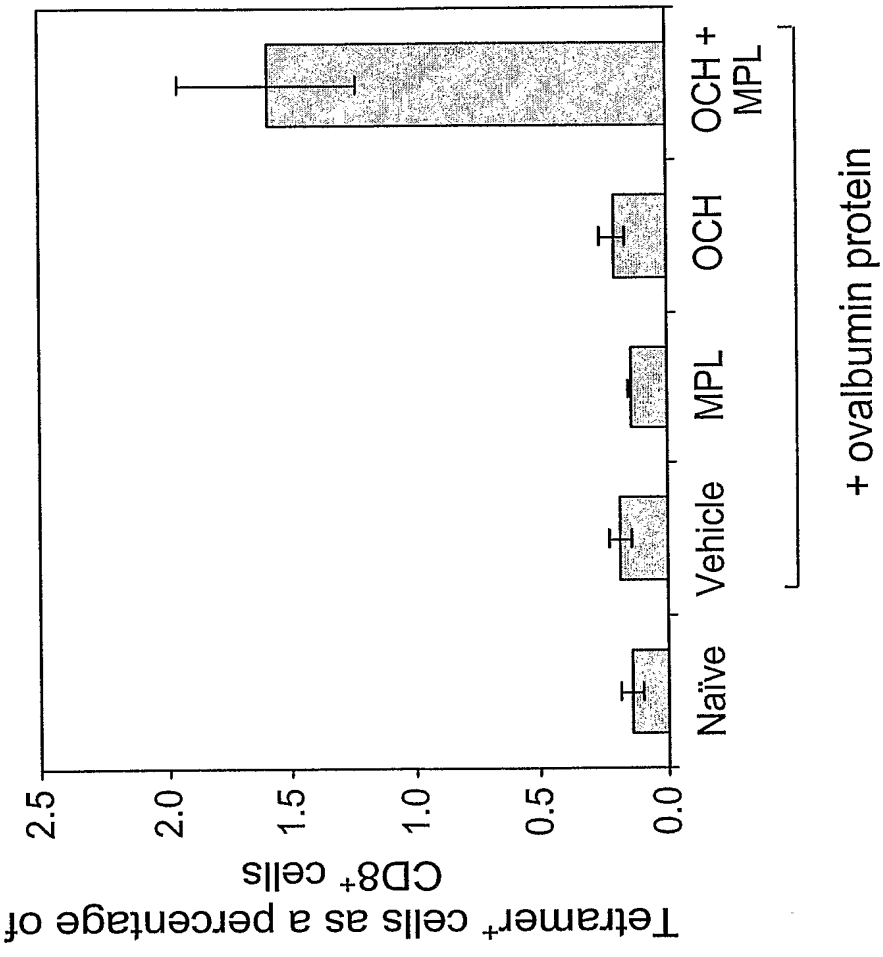

Tetramer+ cells as a percentage of CD8+ cells
2.5
2.0
1.5
1.0
0.5
0.0
Naïve
Vehicle
MPL
OCH
OCH + MPL
+ ovalbumin protein

Figure 14(a)

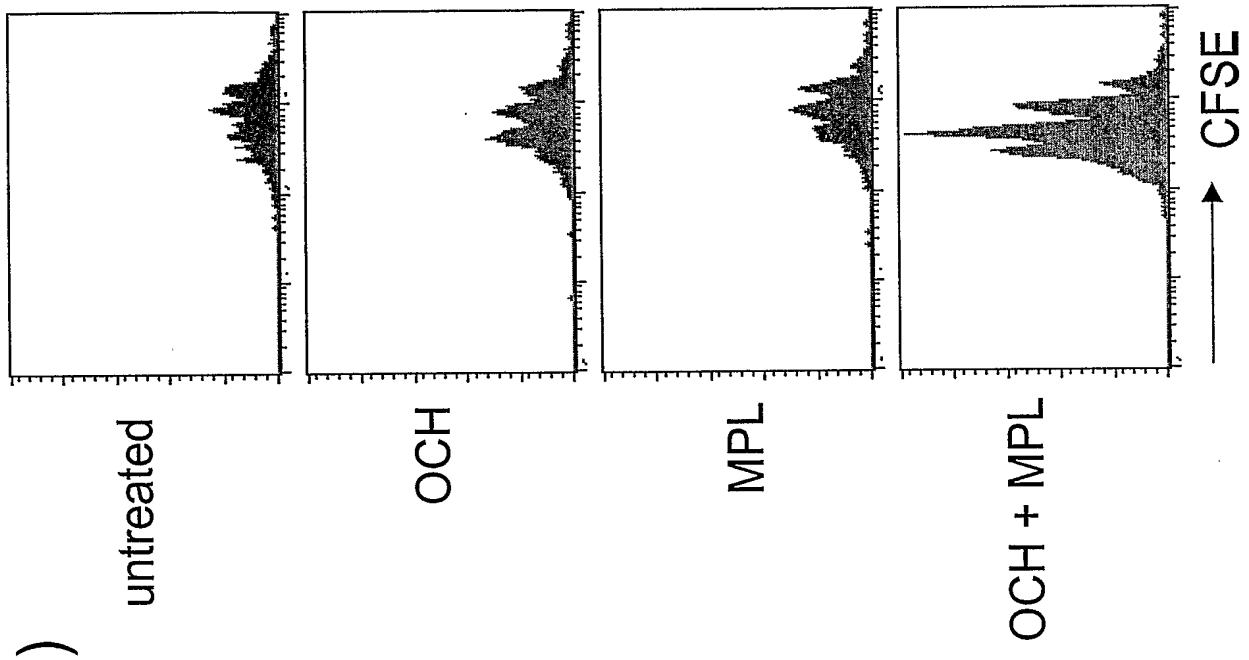
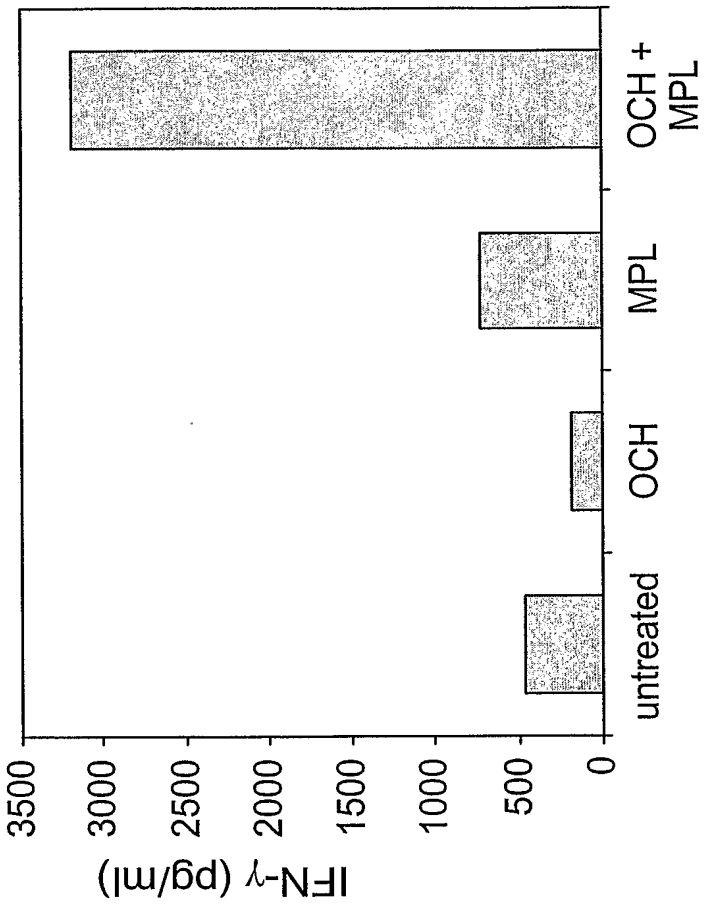


Figure 14(b)


IFN-γ (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
untreated
OCH
MPL
OCH + MPL